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FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE FÍSICA

Decoding the Mechanism of Motor Plasticity During Gait Recovery After Injury in Drosophila melanogaster

Raquel Filipa Gonçalves Fontes Laranjeira

Mestrado Integrado em Engenharia Biomédica e Biofísica
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Dissertação orientada por:
César S. Mendes, Ph.D., Principal Investigator at CEDOC
Alexandre Andrade, Ph.D., Assistant Professor at the Faculty of Sciences of the University of Lisbon

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"I have no special talents.

I am only passionately curious."

Albert Einstein

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Abstract

Coordinated walking in vertebrates and multi-legged invertebrates, such as *Drosophila melanogaster* is controlled by an evolutionarily conserved network capable to control the movement in a fast, stable, and energy-efficient way. But sometimes due to physical challenges, aging and other factors, the reliable and stereotyped walking process can be compromised. To overcome these challenges, some imposed by disease or injury and others by external conditions, the network displays neural plasticity that allows humans, but also fruit flies demonstrate an adaptive motor behavior. Currently, this adaptation mechanism is partially understood and despite the large investments in the field of neurorehabilitation and some improved methodologies, the recovery outcomes are nevertheless considerably variable amongst individuals. This elusiveness is largely due to the absence of an appropriate model to study this process and facilitate the design of approaches to identify new mechanisms required for motor plasticity. Therefore, we aim to establish the fruit fly as a genetic model for neurorehabilitation. For this purpose, we used a detailed gait analysis system, that allows the quantification of several kinematic features with high temporal and spatial resolution. Based on these quantifications, we found that both male and female flies in which the two middle legs were amputated could walk immediately after motor injury, although in a very uncoordinated way. Nevertheless, over the course of a few days, the fruit flies could improve their gait performance gradually, engaging in a more controlled locomotion. Moreover, older flies displayed a consistent decline of motor control and a gradual lack of motor recovery, coherent with observations in elderly patients. Contrary to our initial hypothesis, the gradual lack of motor recovery cannot be explained by their lower exploratory activity.

These results suggest that flies can readjust their motor circuitry upon injury and the motor recovery process is dependent on gender, age and activity.

This study will open new doors to a better understanding of the locomotion process under injury conditions. In future, it may help to develop or improve new therapeutic approaches, like targeted bio-inspired machines, in order to enhance the recovery outcomes.

Keywords: Coordinated walking, injury, neurorehabilitation model, motor recovery, kinematics quantification

Resumo

A coordenação locomotora em vertebrados e artrópodes com múltiplos apêndices articulados, como na mosca da fruta, *Drosophila melanogaster*, é controlada por uma rede neuronal evolutivamente conservada. Esta rede neuronal complexa é formada pelo sistema nervoso, músculos e órgãos sensoriais que interagem constantemente e recebem informações do meio ambiente para ajustar o controlo do movimento, garantindo que a sua execução seja feita de uma forma rápida, estável e eficiente em termos energéticos. No entanto, frequentemente devido a doenças neurodegenerativas, doenças motoras, envelhecimento e outros fatores, esse controlo e, como consequência, a locomoção é seriamente comprometida. Para superar estes desafios, alguns impostos por doenças ou lesões e outros por condições externas do meio envolvente, a rede neuronal é capaz de exibir plasticidade neural, que permite aos seres humanos, mas também às moscas da fruta demonstrarem um processo de aprendizagem, que garante a adaptação motora às novas condições biomecânicas. Atualmente, este mecanismo de adaptação e de aprendizagem é parcialmente compreendido e, apesar dos grandes investimentos no campo da neurorehabilitação e do aperfeiçoamento de algumas metodologias, os resultados da recuperação neuromotora são, no entanto, consideravelmente variáveis entre os indivíduos e, por vezes incompleta. Essa parcial compreesão deve-se em grande parte à ausência de um modelo apropriado para estudar este processo e facilitar o desenho de novas abordagens para identificar os mecanismos necessários para a plasticidade motora. Por estas razões, a proposta do presente trabalho é estabelecer a mosca da fruta como um modelo de neuroreabilitação.

Para além da similaridade existente ao nível dos circuitos neuronais responsáveis pelo controlo da coordenação motora, as moscas da fruta apresentam uma locomoção estereotipada e estável, de fácil caracterização. Para tal, foi usado um sistema ótico baseado na dispersão da luz internamente refletida, que permite caracterizar detalhadamente a locomoção com uma grande resolução quer temporal, quer espacial. Neste sistema foi integrado o *software FlyWlaker* especificamente criado para este propósito. As características cinemáticas quantificadas permitiram determinar a dimensão do tamanho de cada passo, a duração necessária para a execução de um passo completo, a velocidade atingida de cada pata durante cada passo, o tipo de marcha, à coordenação entre patas, a linearidade do movimento de cada pata em relação ao centro do corpo, entre outras. De acordo com estas quantificações, verificou-se que as moscas da fruta de ambos os géneros, nas quais as duas patas do meio foram amputadas, podiam caminhar imediatamente após a lesão motora, embora de uma forma muito descoordenada. Naturalmente, a amputação comprometeu o acoplamento mecânico existente entre as patas, reduzindo a sua coordenação e obrigando um ajuste do tipo de marcha assumido. Antes da amputação, a coordenação das patas podia ser executada segundo quatro tipos de marcha, sendo a mais comum o tripé. No entanto, após amputação este tipo de marcha ficou completamente comprometida e as moscas foram obrigadas a reajustar o acoplamento mecânico das patas, assumindo novos tipos de marcha, característicos dos animais quadrúpedes. Ao longo de alguns dias, verificou-se, de facto, que as moscas eram capazes de melhorar o seu desempenho motor de uma forma gradual, conseguindo ajustar-se às novas condições biomecânicas por forma a ter uma locomoção mais controlada e coordenada. Essa adaptação motora passou por um aumento da linearidade do traçado obtido pelo contacto de cada pata em relação ao centro do corpo. Para além disso, verificou-se um

aumento do *cluster* formado pelas pegadas de cada pata. Este aumento de linearidade e da consistência das pegadas é indicativo do aumento da coordenação motora, como resultado da reorganização e do remapeamento das representações motoras para compensar progressivamente os padrões de locomoção desfavoráveis. E, apesar de se ter verificado que esta adaptação motora era comum em ambos os géneros, constatou-se que as fêmeas apresentavam uma curva de recuperação mais gradual, enquanto os machos apresentavam algumas oscilações ao longo de todo o período de recuperação observado. As possíveis justificações para estas diferenças residem nas diferenças anatómicas, como por exemplo no peso e tamanho. De uma forma geral, como os machos eram menores e mais leves, as propriedades cinemáticas da sua locomoção e, por sua vez, o processo de recuperação motora eram distintas das fêmeas.

Com o objetivo de investigar o efeito do envelhecimento nas propriedades cinemáticas da locomoção e no processo de neuroreabilitação motora, aplicou-se o mesmo protocolo em moscas do género feminino com idade avançada. Neste caso, foi verificada a inexistência de recuperação motora ao longo de todas as idades, consistente com observações em pacientes humanos. Esta inexistência de recuperação foi evidenciada pela dispersão do *cluster* obtido pelas pegadas e pela diminuição da linearidade do traçado de cada pegada, evidenciando que o controlo motor se ia deteriorando ao longo das semanas e que a capacidade de se ajustar às novas condições biomecânicas impostas pela amputação era reduzida. Como possível justificação para estes resultados foi considerada a hipótese de que moscas com idade mais avançada possuíam uma menor atividade locomotora. Esta hipótese não foi totalmente comprovada. Inicialmente, pensou-se que após 1 semana todos os parâmetros iriam diminuir gradualmente. E, apesar de se ter verificado este decréscimo gradual ao longo das semanas, essa diminuição ocorreu a partir das 3 semanas. Na maioria dos parâmetros cinemáticos observou-se a existência de grandes diferenças significativas para as moscas com 3 semanas relativamente aos restantes estágios etários. Posteriormente, constatou-se, de facto, a existência de um pico positivo em todos os parâmetros quantificados para a atividade exploratória. E, contrariamente ao expectável, observou-se que esse aumento era muito mais significativo para moscas com uma idade igual a 3 semanas. Neste estágio etário pareceu existir uma otimização do controlo motor, manifestado por um aumento abrupto da distância total percorrida e, consequentemente da atividade exploratória bem como da velocidade média. Além disso, verificou-se que a recuperação motora após amputação era bem mais eficaz, dado que a maioria dos parâmetros alcançavam os valores controlo, em contraste com os resultados previamente quantificados. Por último, nas moscas com 7 semanas de idade, o efeito da amputação é praticamente nulo, indicando que nesta fase etária o seu normal controlo motor é semelhante à perda de controlo motor induzido pela a amputação das duas patas médias. Adicionalmente, constatou-se em alguns parâmetros a existência de similaridade entre moscas de 1 e 7 semanas. Isto poderia indicar que o processo de aprendizagem motora evidenciada pelas moscas de 1 semana era limitado, mas que, apesar disso, estas tinham uma plasticidade motora mais elevada que as faria ajustar-se melhor às novas condições biomecânicas. E o nível do processo de aprendizagem assemelhar-se-ia à perda de capacidade de aprendizagem das moscas com 7 semanas. Estas não apresentavam qualquer melhoria do controlo motor porque a plasticidade motora seria menor devido à natural senescência. Adicionalmente, o processo de aprendizagem estaria optimizado às 3 semanas de idade. A partir daí, a natural senescência motora em conjugação com a redução da capacidade de aprendizagem causaria a diminuição gradual da capacidade de adaptação.

Em suma, estes resultados sugerem que as moscas conseguem reajustar os seus circuitos neuronais após a lesão motora e o processo de recuperação motora depende do gênero, idade e da atividade locomotora.

Assim, este estudo pode abrir novas portas para uma melhor compreensão do processo de locomoção em termos cinemáticos e, no futuro, pode ajudar a desenvolver ou melhorar novas abordagens, como máquinas biomédicas direcionadas para melhorar os resultados da recuperação motora.

Palavras-chave: Locomoção coordenada, lesão, modelo de neuroreabilitação, recuperação motora, quantificação cinemática

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Acronyms

BG Basal Ganglia

CX Central Complex

CPG Central Pattern Generator

fTIR *frustrated Total Internal Reflection*

MLR Mesencephalic Locomotor Region

MN Motor Neuron

PCA Principal Component Analysis

RF Reticular Formation

CHAPTER 1

Theoretical Contextualization

1.1 CONTEXT PROBLEM

Nowadays, humans suffer from debilitating motor conditions that are caused by stroke, brain injury or limb loss, but also from neurodegenerative pathologies, such as Parkinson's disorder, Amyotrophic Lateral Sclerosis (ALS) (Chio *et al*., 2013), and Spinal Muscular Atrophy (SMA) (Comley *et al*., 2015; D'Amico *et al*., 2011) among others, by descending order of prevalence. These conditions are becoming more frequent in an ageing population and affect millions of individuals worldwide, compromising their voluntary movement coordination, balance and equilibrium during gait (Radovanović *et al*., 2014; Mazzoni *et al*., 2012), having a tremendous social and economic impact. Currently, there are multiple promising strategies for the recovery process or rehabilitation. Some of these strategies include non-invasive brain stimulation (*e.g.* Transcranial magnetic stimulation (TMS)), physical training and exercise, cognitive training, Neuropharmacology, prosthetic limbs and reconstructive surgery and, finally, robotic legs (Claflin *et al*., 2015; Pekna *et al*., 2012; Cramer *et al*., 2011; Kleim, 2011). All therapeutic procedures depend on the remarkable robustness and plasticity of the nervous system in response to debilitating motor conditions. This adaptive plasticity or sensorimotor adaptation is a type of learning, more specifically, motor learning that occurs in the presence of environmental or internal perturbations. These disturbances are interpreted by the nervous system in a complex and intricate process that allows adjusting the motor output and preserve some accurate movements, previously learned (Della-Maggiore *et al*., 2014; Pekna *et al*., 2012).

1.2 PROBLEM

In the debilitating motor conditions referred above, the motor recovery or sensorimotor adaptation can be immediate and spontaneous but over the time, in most cases, the degree of improvement is incomplete (Wahl and Schwab, 2014). This limitation comes from the fact that recovery is a complex process that depends on multiple factors, including the severity of injury, access and response to treatment, training, age, gender and genetic variability. For these reasons, the recovery process and treatment depend on inter-subject variability (Cramer, 2008).

Despite all the knowledge regarding the nervous system, a better understanding of how training and rehabilitation affect the brain remapping is very important. Especially because the current rehabilitative tools (mentioned above) are not known very effective. One of its limitations is due to recovery having a limited range over time (Wahl and Schwab, 2014). A future strategy should include

a new rehabilitation paradigm, which conjugates the natural remapping of the brain and reorganization of neural circuits with novel therapeutic interventions personalized to each clinical case. Genetic tools have emerged as a new approach to this field of research (Adams *et al.*, 2000). Accordingly, recent studies which have taken genetic approaches showed an increased motor recovery, during the neurorehabilitation process. Multiple genes that affect or enhance recovery outcomes in the context of stroke have been identified over the last few decades (see Simon *et al.*, 2012 for details).

Overall, understanding the motor rehabilitation process requires 1) a deep knowledge of neural circuits and mechanisms that underlie neuromotor plasticity, 2) identifying the genes and their role that affect motor recovery process, and lastly 3) the quantification of kinematic changes.

1.2.1 Proposed Solution

To better understand the mechanisms underlying the spontaneous locomotor recovery after debilitating motor conditions, we will take advantage of the fruit fly, *Drosophila melanogaster*. Our proposed solution is to establish this species as a neurogenetic model for rehabilitation. Given the complexity of the neurorehabilitation process, the fruit fly provides an attractive model due to its simplicity and large availability of genetic tools. Since the genes responsible for the development of motor circuits are evolutionarily conserved between arthropods and vertebrates (Adams *et al*. 2000; Mendes *et al*., 2013), motor actions in general, and walking in particular, are governed by the same general rules (Bässler and Büschges, 1998; Pearson, 1993). Both vertebrates (like humans) and arthropods (like flies) are forced to relearn the motor process. So, using the fruit fly, it is possible to better understand the motor recovery process in genetic and neuromotor perspectives. Furthermore, it is possible to quantify the adaptation and the improvement of the fly's motor output through an objective quantification of the outcomes, provided by a detailed gait and exploratory activity analysis (see **Figure 1.1**). Finally, if there is a genetic component that affects *Drosophila* locomotion or recovery, this variance may be relevant for humans. This study may open new doors for the better understanding of locomotion process in terms of kinematics and, in the future, may help develop or improve targeted bio-inspired machines (Mendes *et al*., 2013; Cruse *et al*., 1998).

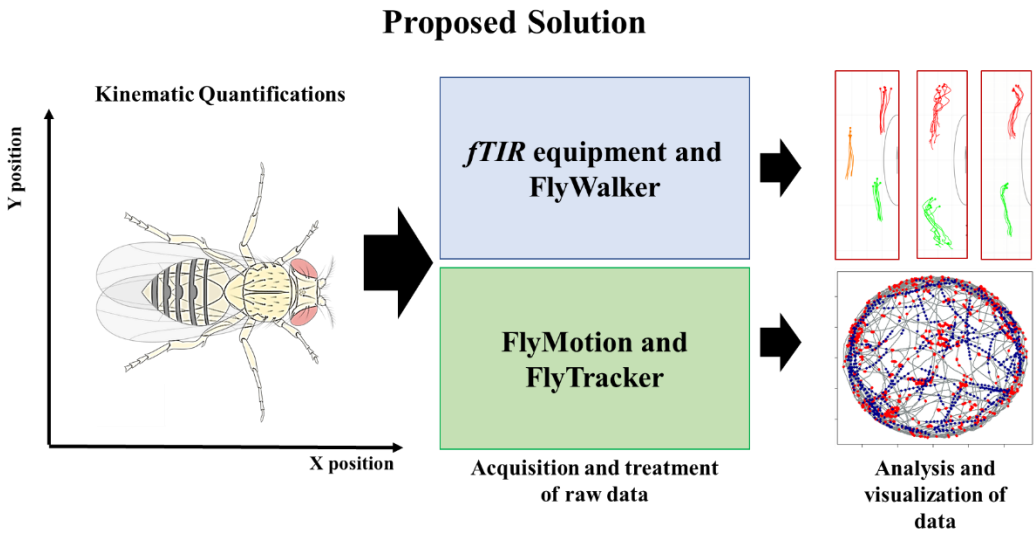

Figure 1.1 Schematic representation of the proposed solution to quantify the adaptation and the improvement of the fly's motor output.

1.3 Motivation

Currently, the knowledge about the contribution of different brain structures responsible for the motor recovery process is only partially understood. The absence of a good neurorehabilitation program and strategies is largely due to the absence of an appropriate model to study this process and facilitate the design of approaches to identify new mechanisms required for motor plasticity.

1.4 Objectives

The main goal of this project was to establish the fruit fly as a model for neurorehabilitation. To achieve this, we used double mid-leg amputations in fruit flies as a method to induce injury and the analysis of temporal kinematic changes to investigate the quantitative influence of some factors (*e.g.* gender, age and locomotor activity) on the mechanisms that underlie the motor recovery.

In summary, the first goal of the present thesis was to test the dependency of gender in motor recovery upon injury. While the second one was to test the ability of older animals to recover upon amputation of their middle legs. In the final and a related goal was to verify if there is a relationship between locomotor activity and the motor recovery in younger and older flies.

1.5 Thesis Outline

The present dissertation is organized into five Chapters. First, the present Chapter exposes the contextualization, the problem and the proposed solution.

In Chapter 2 – Introduction, a brief description of locomotion is made, highlighting the similarities between humans and fruit flies. Some of which resulting from the conserved neural network between these two species. The understanding of this complex network depends on the study of the kinematics of coordinated walking. For this reason, over the years, several approaches were developed. Some of these approaches were described in this chapter, including the assay used in this study.

In Chapter 3 – Materials and Methods, a detailed description of all the relevant materials used in this study is provided, as well as the methodologies used in order to accomplish the initial goals.

In Chapter 4 – Experimental Results, Data Analysis and Discussion, a comparison the motor recovery between male and female flies is presented, as well as between younger and older flies. The locomotor activity was also studied to understand the lack of recovery in older flies.

Lastly in Chapter 5 – Conclusions and Future Work, a general discussion of the results and the proposal of suggestions for future work are made.

CHAPTER 2

Introduction

2.1 Locomotion: human and fruit fly

Through evolution, animals developed anatomical, physiological and behavioral strategies for their survival. These strategies allow the animals to navigate in the environment, escape from predators, find mates and look for food (Dickinson *et al*., 2000). One of the most important strategies to achieve these goals is by the coordinated movement of several multi-jointed limbs leading to locomotion within a substrate. During the evolutionary process, different species adopted different ways of locomotion, such as swimming, crawling, flying or walking. One very important type of locomotion adopted by animals since life became terrestrial was walking.

Walking animals, like humans and the fruit fly, use multi-jointed legs (or limbs) to produce a cyclic behavior, generating alternating activity of flexor and extensor muscles. The step cycle is formed by two distinct phases: (1) the stance (or support) phase, in which the legs contact with the ground, generating forward propulsion of the body and (2) the swing (or transfer) phase that is used to return the legs back to the starting position to generate the next stance phase (Büschges and Gruhn, 2007; Graham, 1985). Despite the similarities, throughout the evolutionary process, the number of multi-jointed legs or limbs necessary to produce the cyclic behavior decreased and the functional structure of the legs in posture (manner how the body is supported by the body appendages) also changed. For this reason, the hexapods (6 legs) have sprawling legs, whilst bipeds (2 legs) are fully erect legs (Reilly *et al*., 2007). The number of legs also influence the kind of gait assumed by the animal. Nonetheless, the chosen gait also depends on body proportions, speed requirements, stability, energy expenditure and required neuromuscular control (Goulding, 2009). The gait stability can be static or dynamic. In general, during locomotion, static stability is guaranteed if the animal's center of mass projection falls within the region covered by the feet on the ground. This configuration is preferable in hexapods. In dynamically stable gaits, like the ones used by quadrupeds and bipeds at higher speeds, there no constraints (Kar *et al*., 2003).

Simultaneous to the similarity existing in coordinated walking, conservation of the motor control process is also seen at the network levels (Büschges *et al*., 2008). In general, this complex network is formed by the nervous system, muscles and sense organs that constantly interact and receive information from the environment to adapt the motor output (Büschges and Gruhn, 2007).

2.1.1 Circuits involved in locomotion

Locomotion is a complex behavior that cannot be explained through a linear process. The locomotion output involves the sequential and coordinated activity of muscles in body appendages or limbs but also

muscles of the trunk in a precise rhythm and pattern (alternating activity in groups of flexors and extensors). To understand how this coordinated locomotion output is processed, the identification of the circuits that underlying to this complex behavior is necessary.

Most of the knowledge about the neural circuits involved in walking behavior came from studies in cats, like in Graham Brown studies' (1911) (Brown, 1911). Brown identified the origin of rhythmic motor output in circuits located in the spinal cord. This set of pre-motor circuits are called central pattern generators (CPGs). Brown saw that cats with their spinal cord transected at the thoracic level (inexistence of higher order commands), showed flexor-extensor hind limb movements. This led to the conclusion that neural networks present in the spinal cord can generate rhythmic movements into alternating flexor and extensor activity in the absence of higher order commands. Until 1980, the cat remained the prevailing model for studying locomotion in vertebrates, but soon after, the researchers start to use new vertebrate models like the lamprey and neonatal rodents. With the advent of molecular genetics in both vertebrates and invertebrates, the possibilities to study the locomotor networks sharply increased. *Drosophila melanogaster* became a very elegant experimental model for the functional analysis of locomotor networks, allowing the specific genetic manipulation of defined small subsets of neurons using the UAS-GAL4 system (Strauss, 2002; Rubin, 1988).

Similar to humans, the network is comprised of motor neurons (MNs), CPGs, sensory neurons and higher control centers in the brain (MacKay-Lyons, 2002; Dickinson *et al*., 2000; Marder and Calabrese, 1996). The connection between the nervous system and muscles is made by the MNs. Each MN, through interneurons, establishes the connection to CPGs, which produce cyclical motor outputs between stance and swing phases (Büschges *et al.,* 2011; Marder and Calabrese, 1996; Pearson, 1993). Although these circuits can act in a self-sufficient manner, the sensory feedback through sensory neurons are very relevant (Bässler and Büschges, 1998). Interaction with the surrounding environment by sensory input allows the continuous adjustment of rhythmic motor pattern. The descending interneurons, which resemble a mesh that keeps all the structures connected, can engage, stop or modulate the cycle frequency of the oscillators. Despite these similarities, controlling the motor output in mammals, especially in humans is made by the motor cortex and the initiation and speed of movement are dependent on mesencephalic locomotor region (MLR), which receives information coming from basal ganglia (BG) and thalamus (Jordan *et al*., 2008; Jordan, 1998). Further, MLR projects to the reticular formation (RF) and the neurons of RF sending information to CPGs, in the spinal cord, that transmits the information to the MNs, to execute the motor action (Le Ray *et al.*, 2011). Whilst, in the fruit fly, the Central Complex (CX) is responsible to control how the CPGs fulfill a motor task (Kahsai and Winther, 2011; Strauss, 2002) through descending interneurons, that provide the interface between the CX and the ventral nerve cord (VNC). In terms of ontogeny and behavioral performance, the BG and CX share extensive similarities (Fiore *et al*., 2015) (see **Figure 2.1**).

In both cases, the conserved network guarantee that locomotion occurs in a fast, stable, and energy-efficient way, while at the same time, provides the flexibility to adapt to changes in environmental conditions. Moreover, during development, motor circuits suffer maturation that results from the need to acquire new motor skills or to accommodate biomechanical changes in a process termed adaptive motor learning (Kiehn and Dougherty, 2013; Zill *et al*., 2004).

The adaptive motor learning process occurs, for example, when humans recover from motor related injuries. In these situations, at the neuronal level, occurs reorganization and remapping of motor representations to progressively compensate the unfavourable motor patterns (Xiao *et al*., 2015; Pekna *et al*., 2012; Pearson-Fuhrhop *et al*., 2009). It would parallel with the extreme case of the removal of the two middle legs in the fruit fly (Isakov *et al*., 2016; Wosnitza *et al*., 2013), where the nervous system undergoes a process of neuronal plasticity, which modifies motor behaviour to cope with the loss of nervous tissue and reduce movement errors (Vasudevan *et al*., 2014). In other situations, like under

changes in walking orientation or gravitational load, the adult fly can induce short and long-term changes in locomotion (Ritzmann and Büschges, 2007; Mendes *et al*., 2014).

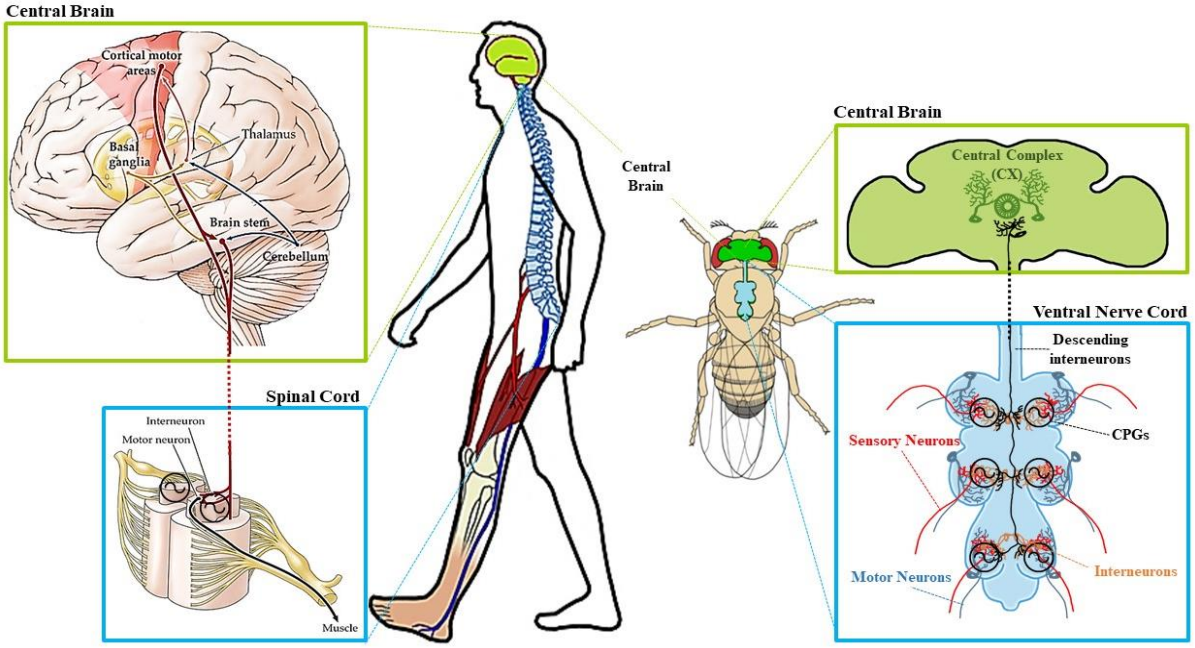

Figure 2.1 Comparison of neural network that control the movement of walking behavior (not to scale). The neural network responsible for the coordinated walking behavior is governed by a conserved neural network, that is comprised of motor neurons (MNs), CPGs, sensory neurons and higher control centers in the brain. Modified from Tucker *et al*., 2015 and Martin, 2003.

2.2 Tracking of fruit fly locomotor activity

It is evident that locomotor activity is an important behavioral outcome underlying the operation of an intricate and complex network. However, it is not totally clear how this network operates to produce locomotion, both in vertebrates and invertebrates' systems. For this reason, the researchers became interested in investigating this behavioral trait in simpler model organisms such as *Drosophila*. This includes courtship behavior, circadian rhythm, learning and memory processes, but also kinematics and coordination during walking behavior. However, during the years, locomotor activity has only been studied indirectly through the quantification of phototaxis/geotaxis (Woods *et al*., 2014; Nichols *et al.*, 2012), sleep (Donelson *et al*., 2012; Gilestro and Cirelli, 2009), courtship and social behavior (Iyengar *et al*., 2012). Diverse assays were developed to quantify the locomotor activity. The most common and simple method allows to distinguish active and inactive flies based on the percentage of crosses in "grid of square" (Connolly, 1966) or the passage between compartments within a labyrinth (Connolly, 1967; Ewing, 1963). With the advent of electronics and image techniques, tracking became more sophisticated and automated.

In the following years, several variants of a small chamber with an infra-red light-gate were developed. The majority of these apparatuses were created to track the locomotor activity in general and the circadian rhythms in particular. The fly activity was recorded when the animal crossed the light-gate collocated in the middle of a chamber. Despite the reliability of this assay, it was unable to quantify the precise fly activity at a given time and the temporal resolution was very low (Martin, 2004).

A higher temporal resolution was achieved by Strauss and Heisenberg (1990). They used a speed motion picture camera at 200 frames/s to study the coordination of legs frame-by-frame. However, the manual frame-by-frame analysis was a time-consuming process.

Later on, the interest in filming more than one fly at the same time was the purpose for the appearance of another setup, the *Locomotron*. This allowed to track 44 flies at the same time (Martin, 2004; Martin *et al*, 1999).

With the advances in computer-assisted electronics, video-tracking started to be more advantageous tracking the locomotor activity. In this context, the assay used in the *Buridan's paradigm*, initially described by Götz, in 1980, was optimized (Colomb *et al*., 2012; Strauss *et al*.,1992; Götz, 1980). In the original paradigm, the tracking of a single wing-clipped fly was made in an open-air arena, placed between two inaccessible black stripes. The short-time window for recording was considered a limitation. For this reason, a new video-tracking paradigm appeared, called *Ethovision*, which gave a complete overview of multiple activity parameters. Furthermore, it allowed to follow freely locomotor activity in "real" time for several hours (for up to 12 h or more) (Martin, 2004).

In 2009, Branson and colleagues followed this approach and proposed an automated, quantitative and high-throughput system for measuring the walking trajectories of a set of fruit flies. Their system was based on machine vision techniques that allowed to track a large group of indistinguishable and unmarked flies, without losing its distinct identities. With this assay, the researchers were able to quantify the trajectories of each fly, including positions and orientations of each frame of video. Furthermore, using machine learning concepts they could to detect automatically the behavior and condensing the resulting trajectories into ethograms (graphs that statistically correlate the social and the individual behavior) (Branson *et al*., 2009; Martin, 2004). However, this kind of assay only allowed to detect the trajectories. Although this was a good measure of locomotor activity, did not allow to obtain the detailed kinematic characteristic parameters of walking patterns.

Despite the advances in the development of locomotor assays becoming increasingly sophisticated, there was still a need for more quantitative and robust methods to analyze the consequences of manipulating locomotor circuits in the fruit fly. All the previous studies, were based either on low-resolution assays for monitoring the average speed of a population and the walking trajectories or on the manual frame-by-frame analysis of videos (Mendes *et al*., 2013).

For these reasons, in 2013, Mendes and colleagues developed a setup based on an optical phenomenon called *frustrated Total Internal Reflection* (*fTIR*). This was used combined with a computer software to automatically detect footprint position and stance phase timing, with high spatial and temporal resolution (Mendes *et al*., 2013). In the same year, Wosnitza and colleagues filmed the flies walking patterns using an assay designated by *tunnel setup*. This new assay uses a single high-speed digital camera to simultaneously film the lateral and ventral view of the walking fly. After, the recorded videos were evaluated frame-by-frame (Wosnitza *et al*., 2013).

2.2.1 High Resolution tracking of fruit fly locomotion

César Mendes and colleagues (2013) developed a kinematic assay based on machine vision techniques. This method combined a high-speed video camera with a custom made-software, allowing the researchers to track the animal's kinematic features with high spatial and temporal resolution, which allowing quantification of many walking parameters.

The operation of this kinematic assay is based on the reflection of light within an optical glass. In this assay, there are LED light sources located at the edges of the optical glass. When the light is turned

on, it starts to travel through the optical glass by an optical effect designated *Total Internal Reflection* (*TIR*).

When the light finds the interface between two different mediums, the glass and the air, whose refractive index is lower, it can be suffering two different optical effects consonant with the angle of incidence. If the angle of incidence is above the critical angle, the light is internally reflected, but when the light founds a denser material, like the tarsus of fruit fly leg, that is in contact with the glass surface, the *TIR* effect is disrupted, forming scatter light by *frustrated Total Internal Reflection* (*fTIR*) (Zhu *et al.*, 1986). The resulting frustrated light is then captured by the high-speed camera. Each video is acquired at 250 frames per second (corresponding to a shutter speed of 4 milliseconds) and the whole video can be analyzed frame-by-frame. In each frame, the *fTIR* effect allows to see the legs of fruit fly that are in stance phase, *i.e.* the legs that are in contact with the optical glass. Furthermore, it is possible to partially see the body due to background light (see **Figure 2.2 A-B**).

Afterwards, the tracking of each tarsal contact and body coordinates are obtained by a custom-made software, named *Fly Walker*. This program evaluates the *fTIR* signals in terms of pixel intensity in each video and provides an array of walking parameters to quantify and compare the walking patterns in different conditions. Here, the footprints and the body center of the fly are tracked throughout of all video frames (see **Figure 2.2 C**) (Mendes *et al*., 2013).

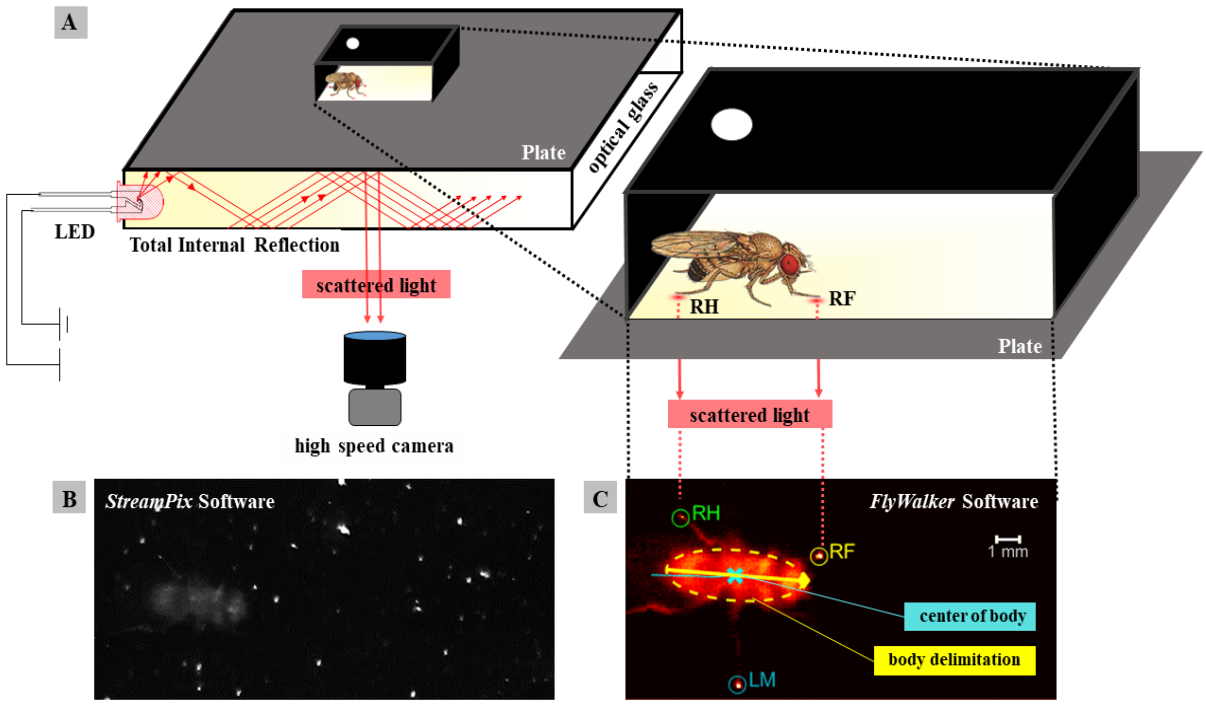

Figure 2.2. Schematic diagram of *fTIR* assay and optical effect, and *Fly Walker* software. **(A)** Detailed description of the *fTIR* assay. The optical glass is covered by a chamber where the fly is placed and let free to walk. The contact of the tarsi with the optical glass, forms the scattered light, which is captured by the high-speed camera. A sequence of multiple images (similar with (B) in *StreamPix* software) is originated, which is then imported to the *Fly Walker* software. **(C)** Image generated by the *Fly Walker* software, where the fly's footprints and body center (blue cross) are tracked throughout the video. The yellow ellipse (dashed line) corresponds to the body delimitation. Adapted from Mendes *et. al*, 2013.

2.2.1.1 Kinematics of fruit fly locomotion

The locomotor assays developed over the years allowed the researchers to observe that fruit flies display extremely reliable, stereotyped, and stable walking pattern (Mendes *et al*., 2013; Wosnitza *et al*., 2013; Strauss and Heisenberg, 1990). It was also seen that walking patterns require different levels of coordination with increasing complexity.

First, on the lowest level of complexity, the walking behavior is guaranteed by the intra-leg coordination that allows the continuous interchange of swing and stance phases for each leg's joints (Berendes *et al*., 2016; Marder and Bucher, 2001).

Second, on the intermediate level of complexity, the interchanges between all legs is guaranteed by the inter-leg coordination, which is governed by several general rules: 1) the forward movement of legs always occurs from posterior to anterior, 2) the swing phase of a front leg does not start until the middle leg behind begins the stance phase (the supporting position) and so on for middle and hind legs and 3) the contralateral legs of the same body segment alternate in phase, which induces the decreasing of retraction time (stance duration) with the increasing of step frequency, while the protraction time (swing duration) stays constant (Cruse *et al*., 2007; Dürr *et al*., 2004; Wilson, 1966).

Finally, both intra- and inter-leg coordination are controlled by the highest level that was responsible for the continuous adaptation of walking pattern in terms of speed and orientation (Berendes *et al*., 2016; Marder and Bucher, 2001).

Most of the kinematic parameters were established by Wilson (1966) and Graham (1972) and summarized by Strauss and Heisenberg (1990), see **Appendix A.1**. But, nowadays, with the *Fly Walker* system, that list of walking parameters was largely increased and it can be divided into three categories: general walking parameters, spatial parameters (which includes coordination variables) and gait parameters (see **Table 1**).

Table 1. List of walking parameters and its units. It can be divided into three categories: general walking, spatial and gait parameters (for details see **Appendix A.1**).

General Walking Parameters		Spatial Parameters		Gait Parameters	
Walking Speed	**(mm/s)**	AEP footprint clustering	**(b.u.)**	Gait index	-
Step length	**(µm)**	PEP footprint clustering	**(b.u.)**	Tripod / Tetrapod / Wave / Non-Canonical Index	-
Step period	**(ms)**	Stance linearity	**(µm)**	Pace / Trot / Walk Index	-
Step frequency	**(cycles/s)**	Stance Straightness	**(µm)**	Tripod duration	**(ms)**
Stance and swing duration	**(ms)**	Footprint alignment	**(µm)**	Inter-tripod transition time	**(ms)**
Swing speed	**(ms)**	Body stability	**(µm)**	Duty factor	-

In terms of general walking parameters, the length, the period and the frequency of each step were quantified. While the step length measures the distance between two consecutive footprints of the same leg, the step period measures the duration of a complete cycle of one leg, comprising the stance and swing durations (Strauss and Heisenberg, 1990) (see **Figure 2.3 A-B**). The step frequency corresponds to the number of step cycles performed during one second. On the one hand, the swing speed measures the average speed of the swing movement. On the other hand, the walking speed corresponds to the average of the total distance traveled per unit of time.

Regarding the spatial parameters, the *Anterior Extreme Position* (AEP) and *Posterior Extreme Position* (PEP) were quantified. The first one corresponds to the first position of the trace (the first contact with the leg and the glass). The second one corresponds to the last position of stance phase (see **Figure 2.3 C**) (Cruse, 1976). Between these two positions, the trace of stance can be obtained. These stance traces represent the stance movement of each leg relative to center of body. Based on these representations, the stance linearity, which is a measure of how much wiggly it is the body center relative to each footprint (considered stationary) was defined. This linearity measure is calculated by the average difference between the real stance trace and the smooth version of the same trace considered. However, it was verified that this parameter is highly dependent on speed, on the size of step, and on the number of steps. So, faster animals, with higher step length or a higher number of steps have straighter stance traces (which corresponds to lower stance linearity indexes) (Mendes *et al*., 2013). To overcome these gaps, another parameter was defined, the stance straightness (Batschelet, 1981). This new parameter results from the ratio between the displacement and the path length of stance traces. Contrary to stance linearity, stance straightness quantifies the linearity of the traces relative to the ideal condition, which would be a straight line between the AEP and PEP footprints position.

Another important feature of locomotion is the coordination. The coordination of walking is measured by the footprint alignment (see **Figure 2.3 (E)**). It is calculated through the mean standard deviation between the front, mid and hind footprints' projections along the displacement axis (Mendes *et al*., 2013).

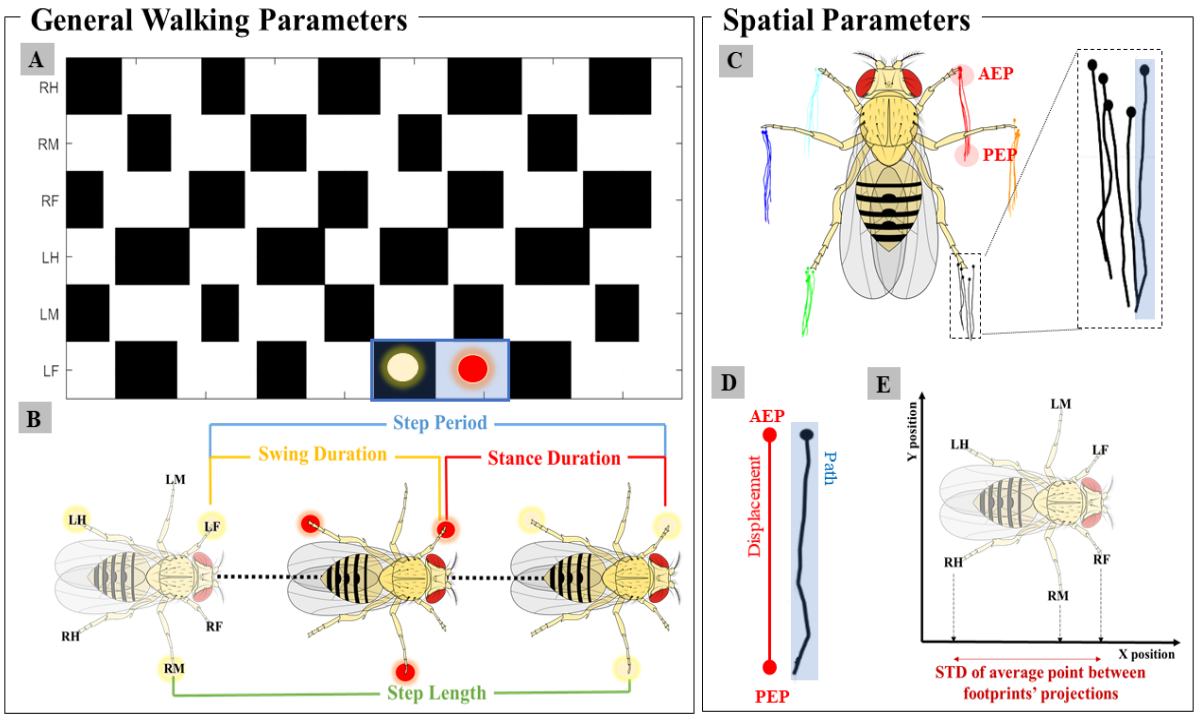

Figure 2.3. Schematic diagram of some general walking and spatial parameters obtained with the custom-made *Fly Walker* software. **(A)** Step pattern, in which are represented the swing (black regions) and the stance (white regions) phases. **(B)** The legs are in swing phase (yellow circles) until contact the glass and generate propulsion of the body (red circles) in the stance phase. The step length corresponds to the distance between consecutive footprints of the same leg. **(C)** Representation of stance traces of each leg, with the identification of AEP and PEP positons. **(D)** The tortuosity of stances traces indicates how much wiggly is the body center relative to each footprint, which is measured by the stance straightness (ratio between the displacement and path values)**. (E)** Method of calculation of footprint alignment, using projection points of footprints along the displacement axis (x position). Adapted from Mendes *et. al*, 2013.

Relative to gait parameters, different kinds of gaits were quantified. For hexapods, the quantification of each used gait was made by two indexes: one quantified the tripod gait and the other one quantified the tetrapod gait (see **Figure 2.4 A**). These two indexes determined the fraction of time that flies spent using each configuration. In the tripod configuration, the fly has three legs in stance phase (for example, the right fore and hind legs and the left middle leg) and three legs in swing phase at any step period; in the idealized tetrapod configuration, the fly has two legs in swing phase and the remaining legs are in stance phase. In this case, the swing phase occurs in two legs of contralateral sides that are offset by one segment (see **Figure 2.4 B**) (Büschges and Gruhn, 2007; Graham, 1985; Hughes, 1952, Mendes *et al*., 2013). Besides these two indexes, were also quantified the non-canonical and the wave indexes. The first one quantified the gait transitions and the second one, the occasional short sequences of pentapod stances (less than 3 steps), in which individual legs swing in a wave-like pattern from front to back (Mendes *et al*., 2013; Wosnitza *et al*., 2013).

For quadrupeds, the inter-leg coordination is defined by three another indexes: pace, trot and walk (Grabowska *et al*., 2012; Büschges and Gruhn, 2007). The determination of these three new indexes was the same used in the characteristic gaits of hexapod animals. The pace configuration is characterized by two legs of the same side in the swing phase, resulting lateral oscillation; in trot, normally, there are two legs diagonally opposite from each other in the stance phase and, finally, in the walk configuration, there are at least sequentially three legs in stance phase.

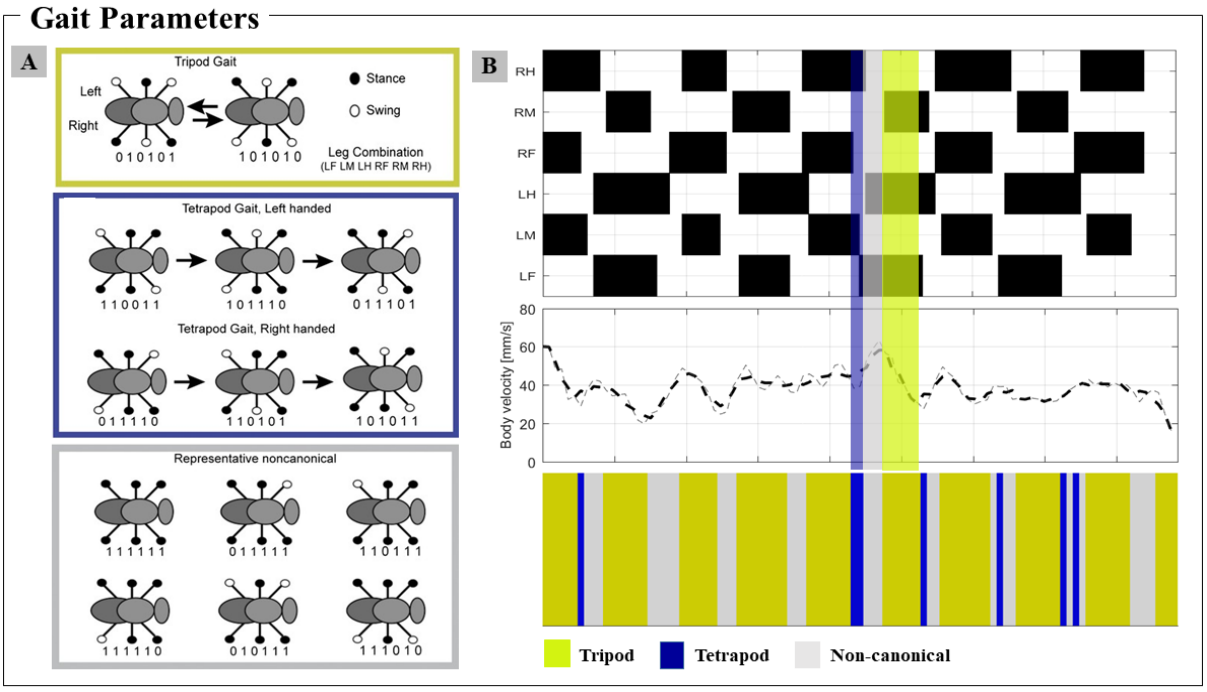

Figure 2.4. Schematic diagram of gait parameters. (A) Representation of different kinds of gait and leg combinations with color and numerical code: black circles and '1' means stance phase and white circles and '0' means swing phase. During the tripod gait, there are simultaneously 3 legs on stance and 3 legs in the swing phase, while in tetrapod there are four legs in stance and 2 in swing phase. This can be either right or left-handed depending on which side is swinging more anteriorly. **(B)** Graph obtained with the *Fly Walker* software. The color code represents the kind of gait assumed by the fly throughout the all movie. For example, this female fly walked at 40 mm/s (average) and the mostly used gait was tripod (green). Furthermore, it is seen that tripod gait corresponds to higher values of body velocity. Adapted from Mendes *et al*., 2013.

This very detailed kinematic information, allows us to fill in the gaps that exist in gait quantification in fruit flies, which in turn will allow a better understanding of the intrinsic factors that are relevant to motor control.

2.2.2 Variables that influence locomotor activity

Over the years of tracking fruit flies' behavior, it was suspected that several factors influence and interfere with locomotor activity. External environmental conditions (*e.g*. light, temperature, humidity and barometric pressure) and internal factors (*e.g*. feeding/starvation, gender and age) (Qiu, 2017; Martin *et al*., 1999).

2.2.2.1 Gender influence

There is a clear sexual dimorphism in *Drosophila*, making it easy to distinguish males and females. On the one hand, females are generally bigger, heavier and have a longer abdomen that ends in an ovipositor (used to lay eggs) with several stripes. On the other hand, males are smaller, have a darker rounded abdomen, and sex combs in front legs. In addition to anatomical differences, brain structure and function dimorphisms were found (Cachero *et al*., 2010). The performance in standard behavioral tests (*e.g*. learning, electric shock avoidance, geotaxis and phototaxis, daily circadian rhythm) also varies with gender (Cong *et al*., 2010; Simon *et al*., 2006). In previous studies were demonstrated differences in the circadian rhythm. According to this study, the female flies displayed higher levels of activity during the evening peak and daytime, while males demonstrated in the night and during the morning peak (Cong *et al*., 2010).

Interestingly, multiple sexual dimorphisms were also found during the aging of fruit fly (Fernandez *et al*., 1999; Le Bourg, 1987). For example, in some previous studies were observed that in the innate ability to escape from the same stimulus, the performance of female flies decline faster than males over 4 weeks (Simon *et al*., 2006; Grotewiel *et al*., 2005; Le Bourg, 2004). These results led Simon and colleagues (2006) to conclude the performance decline in females may reflect significant alterations in learning and memory processes.

2.2.2.2 Age influence

Aging is an inevitable and natural process that is accompanied by the gradual loss of physiological functions (senescence), that results from a complex relationship between environmental and genetic factors (Iliadi *et al*., 2012). Until now, the biology of aging is not fully understood. Considering the conservation of genes and pathways that regulate aging and longevity between multiple species (*e.g.* McElwee *et al*., 2007). The use of model organisms is extremely valuable to a more profound knowledge of these processes. The fruit fly has many advantages to study aging, including a shorter lifespan (around 50 – 80 days) and a good demarcation between development and adulthood phases (Iliadi *et al*., 2012; Leffelaar and Grigliatti, 1983). Also, because the portion of life that animals remain behaviorally functional (function span) can easily be identified in *Drosophila*, the fruit fly is very sought on model (Grotewiel *et al.*, 2005).

The normal aging or functional senescence is related to a progressive behavioral decline (Simon *et al*., 2006; Fernandez *et al*., 1999), especially senescence of motor activity and learning and memory processes (Grotewiel *et al.*, 2005). In 1983, Le Bourg demonstrated that younger flies (1-week-old) were more suitable to move a greater distance than older flies (6-weeks-old) (Le Bourg, 1983). Later, he also found that spontaneous locomotor activity regularly decreased with age, in females, right from the first (Le Bourg, 1987). However, another study, demonstrated that in the first contact with the novel environment, the aged flies (14, 18 and 30 days) are more inactive compared with 3 and 9-days-old flies but, after the adaptation period, the flies from all ages had comparable activity levels. These results led the author to conclude the exploratory activity is not suitable the behavioral senescence (Grotewiel *et al.*, 2005).

In summary, although over the years, it has been demonstrated that gender and age affect the locomotor performance of fruit flies, knowledge about the effects of these factors on the kinematic parameters remains elusive. Taking into account these differences, it is possible to hypothesize that gender and age could influence the walking kinematics before and after debilitating motor conditions.

CHAPTER 3

Materials and Methods

3.1 Locomotor Recovery Experiments

3.1.1 Fly Preparation

In all locomotor recovery experiments, wild-type fruit flies of strain Canton-S (wtCS) were used. The stocks were raised in a 12:12 h light/dark cycle and maintained on standard cornmeal food at 25°C.

Regarding the first goal, after two to three days, some male and female flies from one original stock were transferred to new vials. In these new vials, the flies were able to reproduce. After a few days, if there were eggs, the adult flies were discarded. The vials with eggs were maintained in the above temperature conditions. Approximately 10 days later, the adult flies started to emerge. These adult flies were transferred to another vial with standard cornmeal food, until they reached 3 – 6 days of age. When this age was reached, mated female and male flies were separated to another vial. 30 flies were separated for control (unamputated flies) and around 10 flies for each time point after amputation (15 minutes, 1 hour, 24 and 72 hours and 7 days) of both middle legs at mid-femur to ensuring that leg stump cannot contact the walking surface.

Regarding the second goal (i.e. recovery age dependency), the same protocol was applied. However, when flies reached 3 – 6 days old, only the mated female flies were collected. These mated female flies were maintained until to reach the desired age (1, 3, 5 and 7-weeks-old). When that occurred, the flies were separated into groups. For control (unamputated flies), as well as for each time point after amputation (15 minutes, 1 hour, 24 and 72 hours and 7 days), 30 females were separated.

The amputation procedure was made by anesthesia on a cold plate and with Zeiss Stereomicroscope. The amputation of both middle legs was done at mid-femur to ensure that the leg stumps could not contact the walking surface.

In the first two time points after amputation, each fly was placed in an empty vial. In the other time points, each fly was kept individually in vials with food. In both cases, it was necessary to maintain the vials horizontally to avoid the climbing effort promoted by negative geotaxis. In all cases, before imaging in *Fly Walker* setup, the flies were kept in clean vials for ~ 30 minutes, in order to they were able to clean their legs.

3.1.2 Setup Preparation

One to two hours before imaging the flies, it was advisable to apply in the fly chamber a substance designated by FLUON AD-1 mixed with China ink, to inhibit the flies to walking on the ceiling and walls of the chamber. The image acquisition was made with a *PointGrey* camera (2048 x 1088) at 40

MHz with the following parameters: region of interest (width = 1500px, height = 470px) and the frame rate (250 frames/s). Lastly, it was necessary to clean up the optical glass where the flies walking, with an optic cleaning fluid for digital sensors.

3.1.3 Experimental Procedure

After being kept in an empty vial for ~30 minutes to clean their legs each fly is directly inserted into the fly chamber using a mouth aspirator. The data acquisition is made immediately after the insertion of fly in the chamber, allowing no adapting period to the surrounding environment. The data acquisition last no longer than 1 minute and the fly is again inserted in an empty clean vial to be discarded.

3.1.4 Video Cropping

The temporal cropping of the raw video sequence was made with the *StreamPix* 6.5.0.0 ©2013-2014[1] software in small sequences of .png files (through the delimitation of the first and the last frames). All selected videos contained an uninterrupted walking sequence of 5 step cycles (of each leg), where the fly is walking straight and from left to right. Then, these sequences were load in Image J to be spatially cropped in order to obtain a smaller image containing the sequence of interest. In total were cropped around 772 movies.

3.1.5 Fly Tracking

The fly tracking process was made with the custom-made software, *Fly Walker* (Mendes *et al.*, 2014) written in MATLAB® R2016b (Math Works™). The last sequence of images saved was loaded through this software. Here, the length of the fly is measured directly and introduced in the software settings. Furthermore, the threshold values for legs and body is defined to optimize the auto-tracking process. After the auto-tracking, it is necessary to confirm the detection of legs and the center of the body. If there are incorrections in detection, it is necessary to do hand correction; if not, the evaluation of the movie can proceed. After all this procedure, the evaluation process results in several .png output files and a Microsoft Excel™ file with all parameters necessary for the further analysis. The Excel™ file obtained with the analysis of each movie has one summary line with the principal parameters. These summary lines are all grouped in another file.

3.1.6 Data and Statistical Analysis

The data and statistical analysis were made in MATLAB® R2016b, R and RStudio, GraphPad Prism6 and Statsoft Statistica software.

To see in an automatic and fast way the association between all the walking parameters and speed was made a GUI (Guide User Interface) in MATLAB® R2016b. This software, called

[1] https://www.norpix.com/products/streampix/streampix.php

FlyWalker_PlotGen allowed the input of, in maximum, 6 Excel™ files (each file corresponds to a group) with all parameters and in a few minutes, all the selected variables were represented in scatter plots with or without error bars (see **Figure 3.1**). Beyond the scatter plots, the user could choose area plots or phase plots to represent the data. Furthermore, the user could compare only groups of hexapods/quadrupeds or compare both (each group is represented with the respective color indicated in the interface). From here, it was possible to identify possible relevant variables for further analysis.

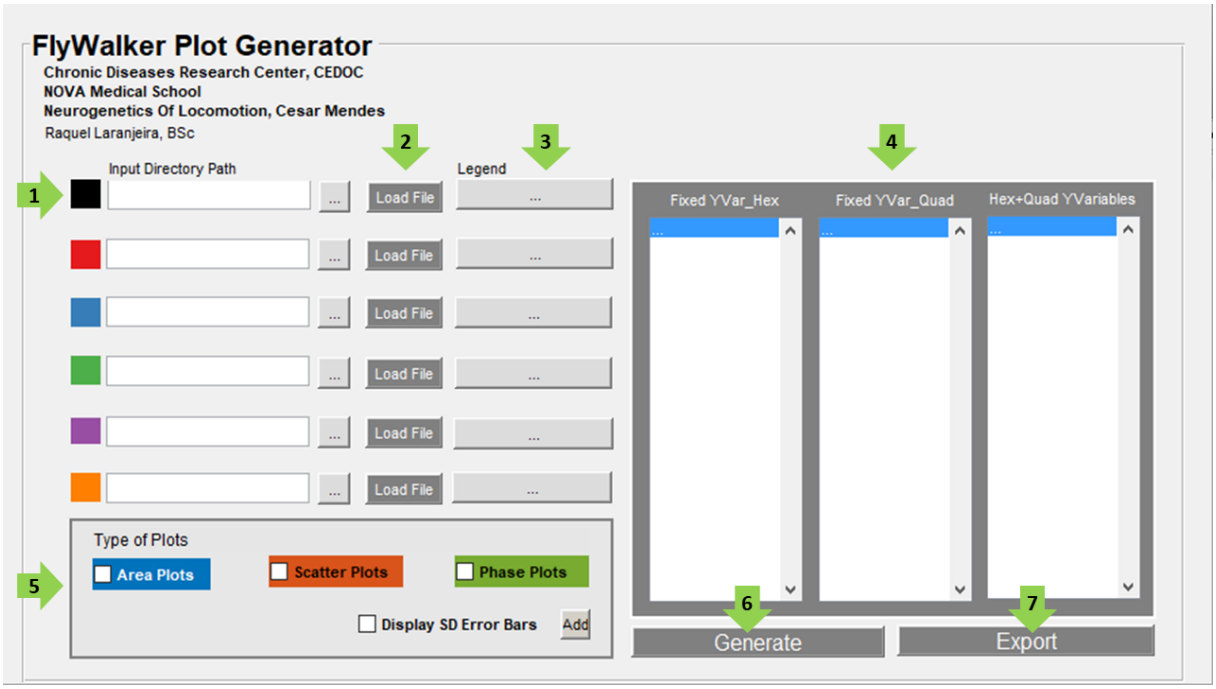

Figure 3.1. Schematic diagram of *FlyWalker_PlotGen* interface. The sequence of use is represented by the numeric green arrows. 1) The first step is to write the path or search the input excel file for all the desired groups. 2) – 3) After, the user needs to press the buttons "Load File" and "Legend": the first allows the software to read all the variables from excel file, including the first cell that corresponds to the name of group and the second display the name of the group. The next step depends on the groups chosen to compare: if all inputs only correspond to hexapods' files, the user press the first column, but if its correspond to quadrupeds' files, the user press the second column. However, if the input files correspond to both groups, the user select the third column. After, the user can choose all the available variables or specific variables. 5) The kind of representation is chosen in "Type of Plots". If the user chose the "Scatter Plots", it is also possible to represent the SD Error Bars. Lastly, 6) the user presses the button "Generate" to display the chosen graphs or 7) save the generated plots in .png files in the working folder.

Based on this first approach, the next step includes the residual analysis in one script written in R. Since many of the measured kinematic parameters vary with speed (confirmed with *FlyWalker_PlotGen*), the R script analyzed the data for these parameters by determining the best-fit regression model for the control experiment and then determining the residual values for each experimental group in relation to this regression model. After, the data were expressed as the difference to the residual-normalized line in boxplots. In order to calculate the statistical differences between the experimental groups, it was necessary to verify whether the initial assumptions of normality and homoscedasticity were fulfilled (using Statsoft Statistica software). The normality assumption was confirmed using the Shapiro-Wilk test (the null hypothesis was not rejected if p-value > 0.05) and the homoscedasticity assumption was confirmed using the Levene's Test. After, whether the residual-normalized data were considered normally distributed and homogeneous, it was used one-way-ANOVA to test the null hypothesis that the means of all groups considered were the same. If the null hypothesis

was rejected (p-value < 0.05), it was performed the Tukey's post hoc test between each group. Whilst in the case of the assumptions was not fulfilled, non-parametric tests were performed. In these cases, for comparisons of more than three groups, it was used Kruskal- Wallis analysis of variance (ANOVA). If the null hypothesis that median of all groups was the same was rejected (p-value < 0.05), the Dunn's post hoc test was posterior applied to see the significant differences between each group.

All the graphic representations were made with scripts written in R and in GraphPad Prism6.

3.2 Activity Experiments

3.2.1 Fly Preparation

In all activity experiments, wild-type fruit flies of strain Canton-S (wtCS) were used. All the stocks were raised in a 12:12 h light/dark cycle and maintained on standard cornmeal food at 25°C. After two to three days, some male and female flies from one original stock were transferred to five new vials. In these new vials, the flies were able to reproduce. After a few days, if there were eggs, the adult flies were discarded. The vials with eggs were maintained in the above temperature conditions. Approximately 10 days later, the adult flies started to emerge. These adult flies were transferred to another vial with standard cornmeal food, they reached 3 – 6 days of age. When this age was reached, it was only collected the mated female flies to another vial. These mated female flies were maintained until to reach the desired age (1, 3, 5 and 7-weeks-old). When that occurred, the flies were separated into groups. For control (unamputated flies) were separated 5 groups of 5 flies. For each time point after amputation (immediately after, 3 and 7 days after) were separated 5 groups of 5 flies. Each group of 5 flies was briefly anesthetized with ice before being filmed.

3.2.2 Setup Preparation

The setup used for activity experiments was the *Fly Motion* (see **Figure 3.2 A**). This setup is formed by one arena with sloped walls to prevent that flies remaining in that region (Simon and Dickinson, 2010). The arena was placed in a white platform that was illuminated by white LEDs, that guarantee a uniform illumination. This assay is also equipped with removable cardboards that avoid the entrance of another kind of light and maintain the external conditions stable. Before the tracking, it is necessary to confirm whether arena is on collocated the right position above the platform and if the detection is within the region of interest, previously defined in FlyCapture2 ©2017 software. The capture was made with a camera Point Grey Flea3 (2080x1552) in JPEG files with a compression equal at 75 (see **Figure 3.2 B**).

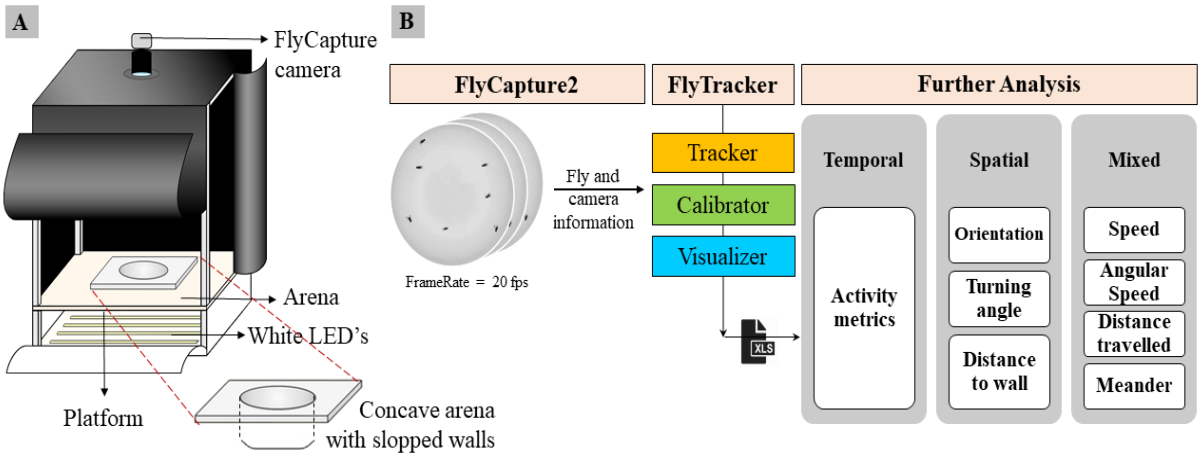

Figure 3.2. Schematic diagram of the *FlyMotion* assay and experimental procedure. (A) The activity tracking of flies was made in *FlyMotion* setup that is composed by one arena placed above a platform uniformly illuminated by white LED's. The spontaneous movement of flies was captured by the camera**. (B)** The experimental procedure starts with setting the camera and fly parameters in FlyCapture2 software. After the output file was introduced in the *FlyTracker* software composed by: Tracker, Calibrator and Visualizer. The output of the analysis is obtained a .xls file with some automatic calculated metrics.

3.2.3 Experimental Procedure

After the setup preparation, each group of 5 flies was anesthetized in ice for 1-2 minutes in order to allow its placement in the arena. Once placed in the arena, the white LEDs were turned on. After the period of adaptation (around 5 minutes), the recording of locomotor activity starts during 15 minutes at 20 frames/s. After that period, the recording was stopped and the used flies were again anesthetized in ice to be inserted into the vial to the remaining experiments. In total were filmed 40 movies.

3.2.4 Fly Tracking Analysis

The analysis of fly tracking was made using the Caltech *FlyTracker* software[2] (Eyrún Eyjólfsdóttir and Pietro Perona, Caltech). This software allows to track the position and the orientation of several flies reconstructing their movement, but also gives information about velocity, size, wing- and leg positions, without losing flies identities throughout the video acquired.

The analysis procedure includes two phases: in the first one, the user introduces the .avi file through Tracker GUI (see **Figure 3.2 B**) in order to do the calibration using the Calibrator GUI. Here the user defines the acquisition frame rate and the diameter of the region of interest. In the experiments, the acquisition frame rate was 20 frames/s and the region of interest was a circle with 58 mm of diameter. After, the user confirms the number of flies detected by the software and adjust the threshold values for background and body detection. In all the experiments, the background and body thresholds were adjusted in order to detect only the body, ignoring legs and wings detection. Then the analysis is performed and the results are saved in one excel file. When the tracking of each fly is finished, it appears in the Visualizer GUI. Here, the user can see the trajectories performed by each fly throughout the movie and whether necessary correct manually the identities. It is also possible to see the calculated variables over all the frames.

3.2.5 Data and Statistical Analysis

Data analyses were done using MATLAB® R2016b, R and RStudio, GraphPad Prism6 and StatSoft Statistica software. Like it was said previously, from *FlyTracker*, several parameters were automatically extracted, such as x, y positions of fly in the arena. From here, it was possible to calculate three kinds of parameters: Temporal, Spatial and Mixed parameters (see **Figure 3.2 B**).

In terms of mixed parameters, the speed was automatically obtained with the *FlyTracker*, while the total distance moved and the meander were posteriorly added. The total distance moved was calculated as a sum of all distances between each frame (see **Figure 3.3 A**). The meander, which is a measure of the straightness of trajectory, is the ratio between the Euclidean distance and the total path length. Both distances were calculated considering three consecutive positions (see **Figure 3.3 A**) The Euclidean distance was calculated between the first and the third positions, while the total path length was calculated as the sum of the two step lengths (Euclidean distance between two consecutive positions) (Edelhoff *et al.*, 2016).

Regarding the spatial parameters, it was considered the turning angles and the centrophobism indices. The first one was calculated by *FlyTracker* and corresponds to the difference between followed

[2] It is available for download at http://www.vision.caltech.edu/Tools/FlyTracker/.

orientations. The second one corresponds to the preference of fly staying in center or periphery of the arena, also called thigmotaxis (White *et al*., 2010; Besson and Martin, 2005; Götz and Biesinger, 1985). This parameter was calculated based on the radial position of fly comparing with the external radius ($r_e = 29\,mm$) or with the internal radius, previously defined as $\sim 0.707 \times r_e$. If the radial position of fly was between both radius, the centrophobism is considered unitary, whilst if the radial position of fly was within the intern circle, the centrophobism is equal to -1. On the border of the two regions, the centrophobism was considered null (see **Figure 3.3 A** and **B**). Quantifying the number of unitary values, it was possible to define a percentage of phobism to center of arena.

Finally, for the temporal parameters, it was considered the activity metrics. These were calculated from speed thresholds, that were empirically calculated by averaging of several periods of time (see **Figure 3.3 A** and **C**). In each frame, all the flies present an instantaneous speed that in comparison with threshold values, previously determined, allowed to define whether the fly is walking or is at rest. So, if instantaneous speed was above the higher threshold (4 mm/s) the fly was classified as walking, but if it was below the lower threshold (2 mm/s) the fly was classified as at rest. However, sometimes the instantaneous speed assumed values between the two thresholds. In this situation, it is considered that fly maintained its previous classification until the second threshold was crossed (Colomb *et al*., 2012; Martin, 2004). All the additional parameters were posteriorly and automatically calculated with a custom-made GUI written in MATLAB® R2016b.

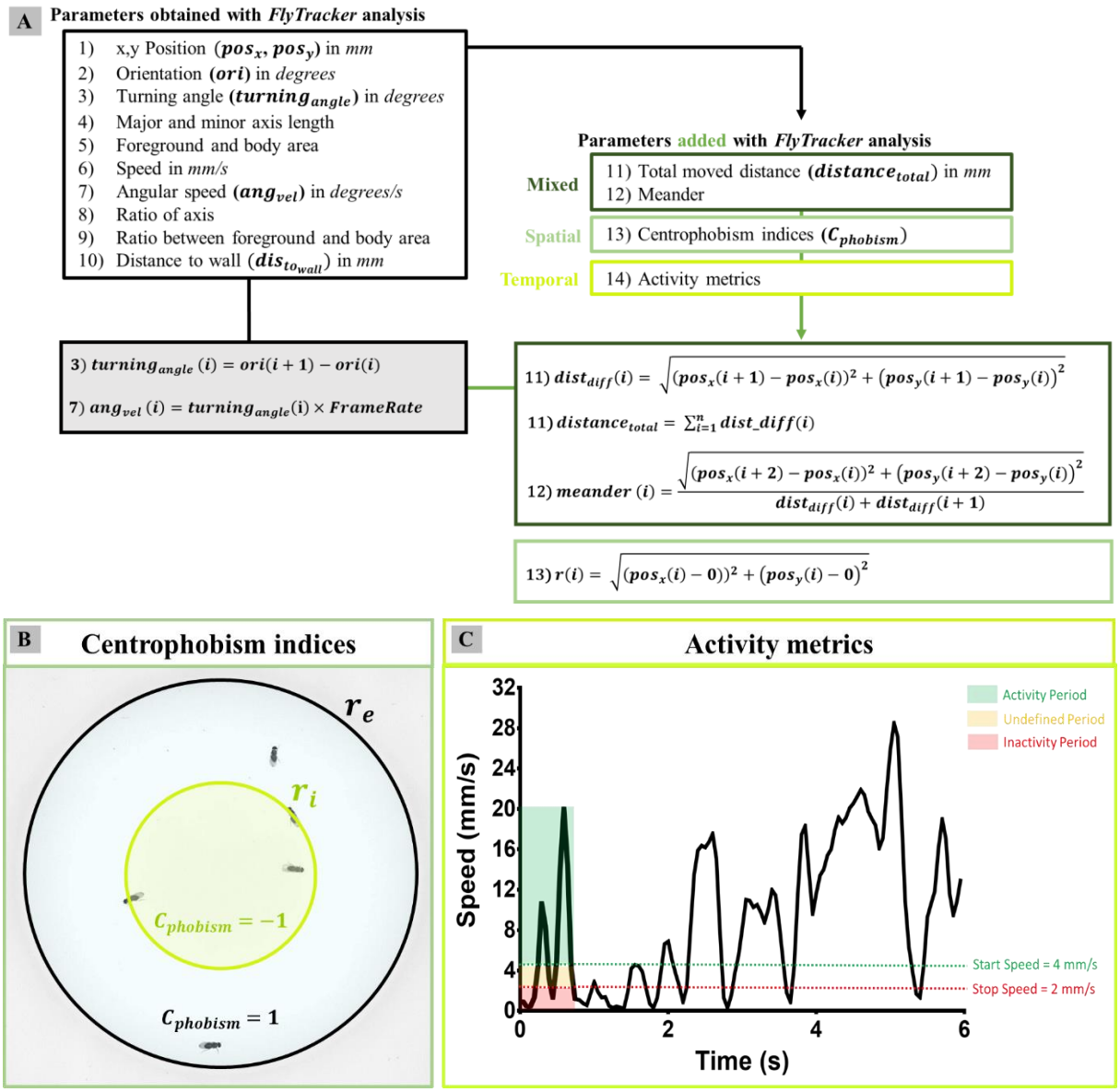

Figure 3.3. Schematic diagram of parameters automatically obtained using the *FlyTracker* software and the further addition of new parameters. (A) Flow chart that indicates the existing parameters given by the software and the parameters added for further analysis. The parameters added can be classified as mixed, spatial or temporal parameters. Their calculation was represented by the respective formulas. **(B)** Representation of how the centrophobism of each fly in each frame is classified. **(C)** Representation of speed during a period of 6s from the whole video (15 minutes at 20 frames/s) and demonstration of classification of periods of activity and inactivity based on the speed thresholds.

CHAPTER 4

Results, Data Analysis and Discussion

4.1 Locomotor Recovery: gender effect

It was previously seen that there are multiple dimorphisms during the performance of a few standard behavioral tests between female and male flies (Cong *et al*., 2010; Simon *et al*., 2006). But the understanding how this intrinsic factor influences the recovery outcomes, after motor debilitating conditions, remains elusive until now. In the present thesis, we aim to test the gender effect on locomotor recovery. For that, we used fruit flies with both middle legs amputated. But first, it was necessary to see the normal kinematic differences in non-amputated flies from both genders. For this comparison, we filmed 30 flies of both genders with 1-week-old, using the *fTIR* assay, that allowed the quantification of the walking behavior with high temporal and spatial resolution, through detection of the scattered light resulted in disruption by the *Total Internal Reflection* process, by the contact of fly legs in the optical glass. The further analysis of walking pattern was made with the custom-made software, the *Fly Walker*. With this software, it was possible to obtain several walking parameters that describe the kinematic changes throughout the walking behavior. However, the association and the relevance of these parameters in the walking behavior requires further analysis. The first analysis was used to see the association between all of them using the correlation coefficients' analysis. This indicates the association strength and the kind of association. From the correlation analysis, some useful information was obtained. For example, there is a greater positive association between the transition time and duty factor parameters, but also with swing duration and tetrapod index. According to these positive correlations, the transition time (or inter-tripod time), between each considered tripod configuration, increases with the increasing of duty factor, step period (especially, the swing duration) and tetrapod index. In non-amputated flies, if the transition time increases, it means that the walking speed decreases (indeed, the inter-tripod time negatively correlates with speed). Thus, for lower speeds, besides the increase of duty factor, the swing duration also increased and the tetrapod gait becomes the preferable gait (see **Figure 4.1 A**). Contrariwise, the negative associations are most evident between speed, duty factor and transition time, tripod index and tetrapod index, tripod index and transition time. Once more, it is evident that some of these associations are redundant. In order to reduce the redundancy and to understand how walking pattern, described by these parameters, may be affected by gender, we performed the Principal Component Analysis (PCA) to our dataset. It was used to summarize the information described by the multiple walking parameters obtained, reducing its dimensionality (removing the noise and the redundant data) by the transformation of the initial variables into a new small set of variables, without losing the most important information in the original dataset. The new variables, called principal components, corresponding to a linear combination of the originals, that only accounts for most of the variance in the observed variables (Jolliffe and Cadima, 2016; Abdi and Williams, 2010; Ringnér, 2008). The number of these new determined variables is the same from the original variables and for this reason, it is necessary previously to determine the number of new variables that are relevant for the study. Using

the *Scree Plot* (a plot that represents the amount of variation (eigenvalue) by each component) and considering the cut-off point (equal at one), the components whose eigenvalues are above the unitary value are considered relevant for the study. In this case, at least five components could be considered, but the graphic representations were only considered the first two components (see **Appendix A.2**).

So, with this analysis it was possible to summarize the results of correlation analysis and determine the relationship between walking parameters of female and male flies. However, it was important to keep in mind that this kind of analysis does not optimize the differences between groups compared. Even so, it allowed to compare visually males and females using the totality of the metrics computed, through biplot representation (see **Figure 4.1 B**). These differences were immediately evident by t separating centroids that result from the different contribution of each parameter represented by the arrows. The contribution is given by the length and direction of each arrow. The angle between each arrow represents the correlation. In this way, if the angle is around 90º, there is no correlation between the considered variables (*e.g.* stance straightness and step frequency, inter-tripod time and duty factor). This can also be confirmed in the correlation matrix (see **Figure 4.1 A**). In this case, for example, the transition time and duty factor are highly positively correlated and, for this reason, the two vectors form a null angle. It is also important to refer that in the biplot representation it only represented the variables, whose *squared cosine* (that indicates the importance of each variable in each principal component) were higher than 0.5 (see **Appendix A.2**)

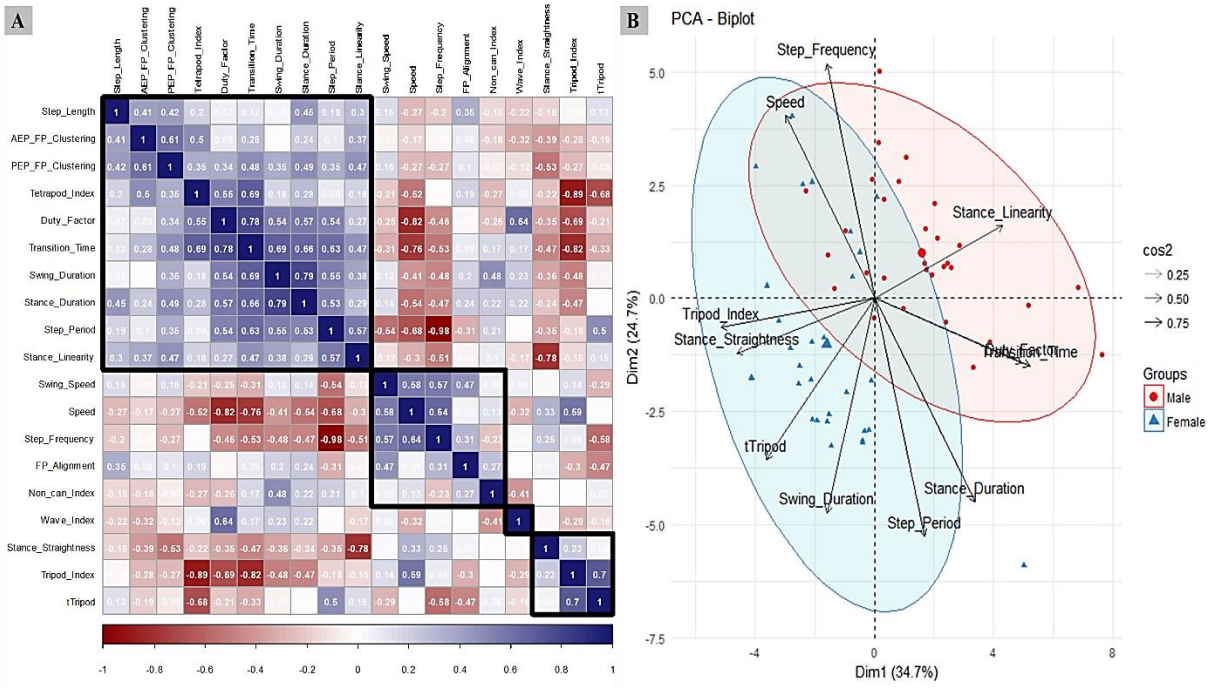

Figure 4.1. Representation of the correlation matrix of all considered walking parameters and biplot representation of PCA. (A) Calculation of correlation coefficients between all the walking parameters. The positive correlation is represented by the gradient in blue and the negative values are represented by the gradient in red. The higher correlation values correspond to the stronger colors. The values within each square represent the correlation coefficient between the intersected variables. The black square indicates variables clustered according to the positive correlation values. The white squares represented insignificant correlations in confidence level of 95% (p-value > 0.05). **(B)** Representation of principal components in a biplot and grouped by gender. The 1st principal component (Dim1) explains 34.7% of total variance, whilst the 2nd component (Dim2) explains the remaining that is not explained by the 1st.

In summary, the walking coordination and gait features (*e.g.* stance straightness, duty factor, tripod index and transition time) contribute to the first principal component, which is explained by 34.7% of the total variance. While the general walking parameters contribute to the second principal component (24.7% of the total variance). Both components explained almost 60% of all variance. Most of the differences between female and male flies come from the first component. So, it is possible to hypothesize that the major walking differences resulted in the parameters that highly contributed for the first principal component.

4.1.1 Comparison of locomotion kinematics in unamputated fruit flies of both genders

Once most of the walking parameters vary with speed (Mendes *et al*., 2013), the analysis proceeds for statistical modulation to calculate the linear or nonlinear regression of all parameters and for both genders. Considering the correlation matrix, it is verified that there are a few stronger correlations between all the variables and the speed, but most of them had a poor correlation, which indicates the absence of a linear dependence or a very weak linear dependency. With these results, it was confirmed that some of them had a linear dependence, but the other had power (*e.g.* Step Period and Stance Duration) and logarithmic dependency (*e.g.* Stance Linearity and Stance Straightness). Regarding the general walking parameters, despite the differences, these parameters had a similar trend for both genders. It confirmed that with increasing the speed the step frequency, swing speed and step length also increasing (see **Figure 4.2 A-C**, the females are represented in pink and the males in blue). And this increase is very similar in both genders, but in swing speed parameter, it was verified that males assumed higher values to perform the swing phase at higher speeds compared with females (see **Figure 4.2 B**). This result can be related to the swing duration result. It was verified that in males, the swing duration is almost constant and independent of velocity. Thus, the step duration essentially only results from the stance duration. Whilst, in female flies, the swing duration tends to suffer a decreasing with the speed increasing (see **Figure 4.2 E-F**).

Regarding spatial parameters, the first difference comes up in PEP Footprint Clustering (see **Figure 4.3 B**). The trend of this parameter with speed became inverse considering gender. In females, the PEP positions became more clustered with increasing of speed (Mendes *et al*., 2013), whilst in males, the same parameter became less clustered with the increase of speed (see **Figure 4.3 B**). These results suggest that with increase of speed, the female flies tend to be more careful in the position of its legs, and for this reason, the steps can be more spatially restricted. In contrast, the males tend to be less spatially restricted and not presenting a higher control in the position of its legs as speed increases. These results are in concordance with the higher values in the stance linearity (or the lower values in the stance straightness). Once the males assumed higher values in stance linearity, the stance traces are less straight, indicating that the body movement is wobblier relative to each footprint (see **Figure 4.3 C-D**). The same trend is observed for AEP Footprint Clustering and the Footprint Alignment (see **Figure 4.3 A, E**). This last parameter can be explained by the follow-the-leader rule. This indicates that in hexapod animals the contact of leg in the stance phase tends to occur close to where the immediately anterior ipsilateral leg made contact (Mendes *et al*., 2013). This is a measure of coordination that depends on permanent sensorimotor adaptation. For this reason, during walking the mid and hind leg footprints fall close to where the front leg was placed. For female flies the footprint alignment values increase with speed, indicating the worst control of coordination as speed increase. In contrast, the males present minor

values for higher speeds. In other words, as speed increases the control of coordination becomes more restricted, allowing better coordination.

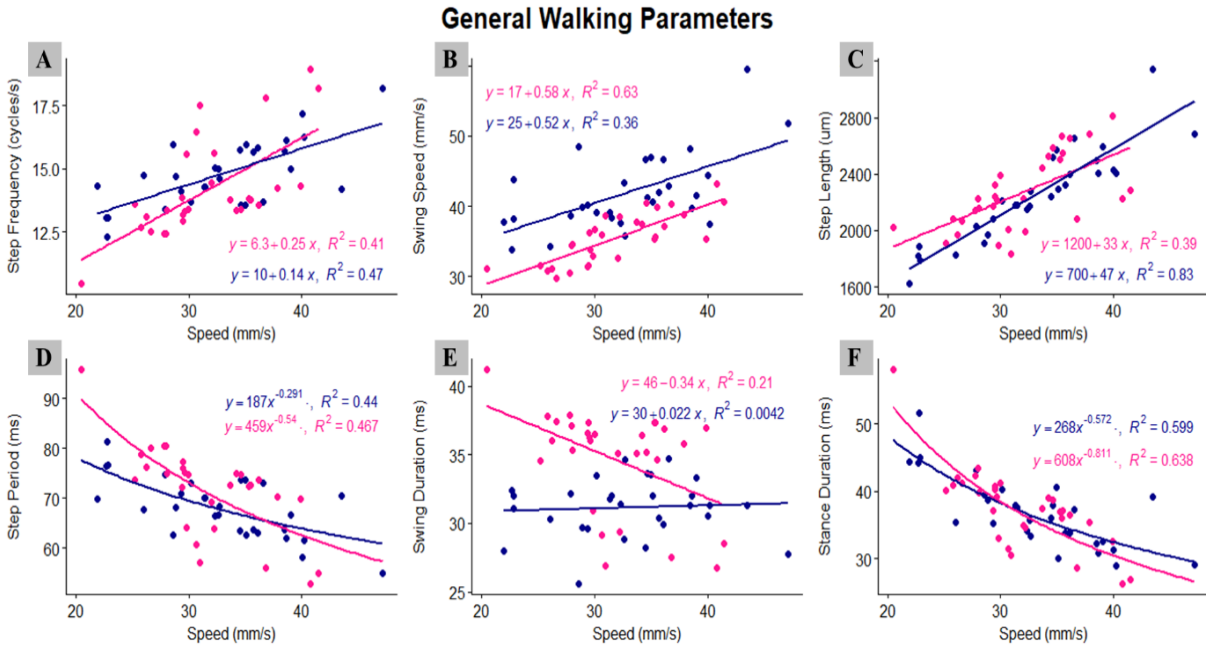

Figure 4.2. Representation of relation of all parameters with speed. (A–F) Quantification of General Walking Parameters with the speed and its (A-C, E) linear or (D, F) non-linear regression (specifically, power regression). Denotation of respective equation and coefficient of determination, R^2. Each graph results of at least 30 videos from 10 different flies of both genders.

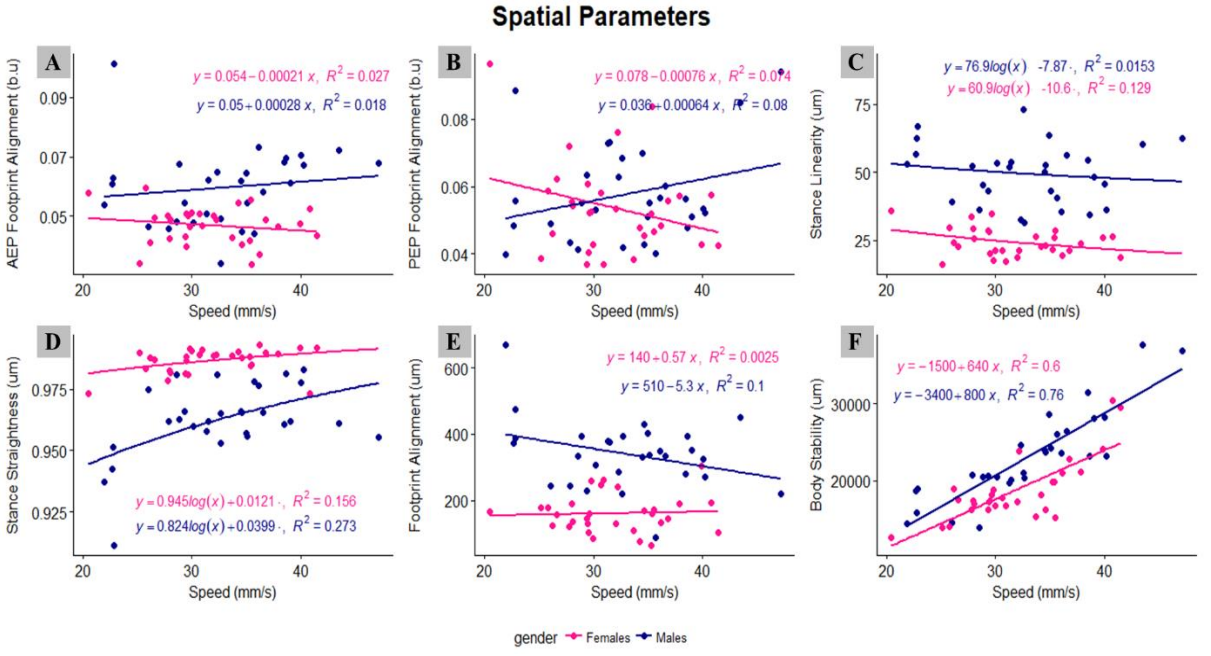

Figure 4.3. Representation of relation of all parameters with speed. (A–F) Quantification of Spatial Parameters with speed and its (A-B, E-F) linear or (C, D) non-linear regression (specifically, logarithmic regression). Denotation of respective equation and coefficient of determination, R^2. Each graph results of at least 30 videos from 10 different flies of both genders.

Lastly and considering the gait parameters, the kind of gait assumed by the animal depends on speed, but, according to these results, it also depends on gender. According to the speed, the percentage of gait varies. In males, with the increase of speed, the tripod gait increases, but for higher values (above the 45 mm/s), it suffers a slight decrease, whilst the tetrapod gait increases and the wave index decreases (see **Figure 4.4 D, E**). In females, unlike males, the tripod gait always increases and the tetrapod index decreases with speed (see **Figure 4.4 B, C**). Furthermore, it is noticeable that males use less tripod gait and more wave gait for most of the speeds. In addition, the males can assume higher values of the average speed compared with females (the maximum value of the speed range for males is 50 mm/s) (see **Figure 4.4 A**). Most of these differences can be seen in the representative color codes (see **Figure 4.4 C, E**). The female color code is practically full of green regions, which indicates a high usage of tripod gait, especially for higher speeds. Even so, there are some percentage of non-canonical gait (grey regions), mostly used between the transition of two tripod gaits. In this case, the non-canonical gait also includes the wave gait.

In contrast, the male representative color code is practically full of blue and green regions, almost by the same percentage. But, in males, the percentage of usage of each kind of gait seems to be more balanced. And the repetition of tripod cycles seems to be longer. In other words, the male flies take longer until to start a new tripod cycle. And for this reason, the inter-tripod time is expected to be longer. Similarly, with females, the male flies used the tripod gait during higher speeds and the tetrapod gait during the lower speeds.

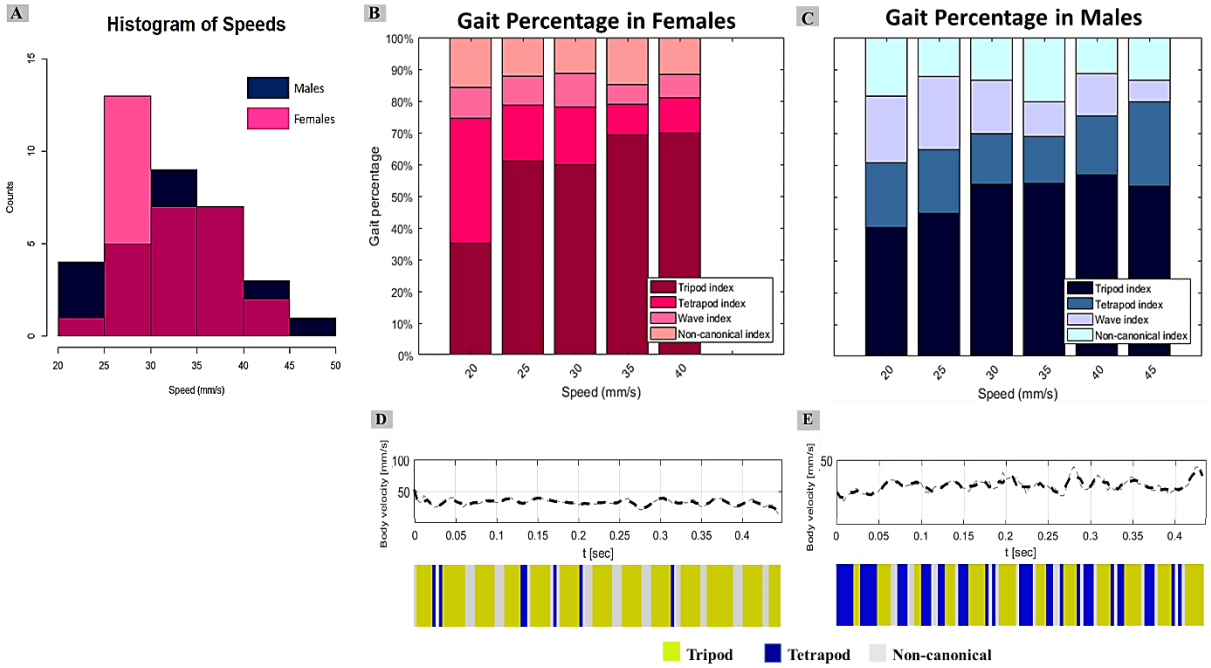

Figure 4.4. Comparison of percentage of gait used by flies according to the speed. (A) Speed histogram of 30 movies recorded for both genders divided into 5 mm/s bins. **(B), (D)** Representation of percentage of gait for each 5 mm/s bin for unamputated (B) female and (D) male flies. Each bar plot was obtained from 30 videos for each gender. **(C), (E)** Two representative color codes representing the kind of gait used over the walking duration and the respective body center velocity (mm/s) for (C) female and (E) male unamputated flies.

Once dependence of all parameters with the speed was verified, it was necessary to remove this dependency to identificate better the differences between both genders. Regarding this, it was performed a residual analysis. After the determination of best-fit regression model for the reference group (female

flies), it was necessary to calculate the residual values for males' data in relation to the specific regression model. With the residual normalized values was performed normality and homocedasticity's tests to in further analysis to know how kind of tests to perform. If these two initial assumptions were verified, the two groups were compared with a parametric test, in specific with one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. But, if the assumptions were not verified, the two groups were compared with the Kruskal-Wallis followed by Dunn's post hoc test.

Considering this analysis, it was possible to confirm some differences, founded in the previous analysis, between each group independently of speed. In general walking parameters, especially in swing speed and swing duration there are significant differences. Independently of speed, the swing speed values are significantly higher for males and consequently, the swing duration is smaller compared with females (see **Figure 4.5 B, E**). These results indicate that male flies perform the swing phase faster than females. In other words, the males' legs spend less time in the air during the swing phase, assuming higher velocities. The first hypothesis considered to justify this was the anatomic differences. In other words, the male flies can assume higher values of swing speed because they are shorter and lighter, which can induce alterations in locomotion kinematics.

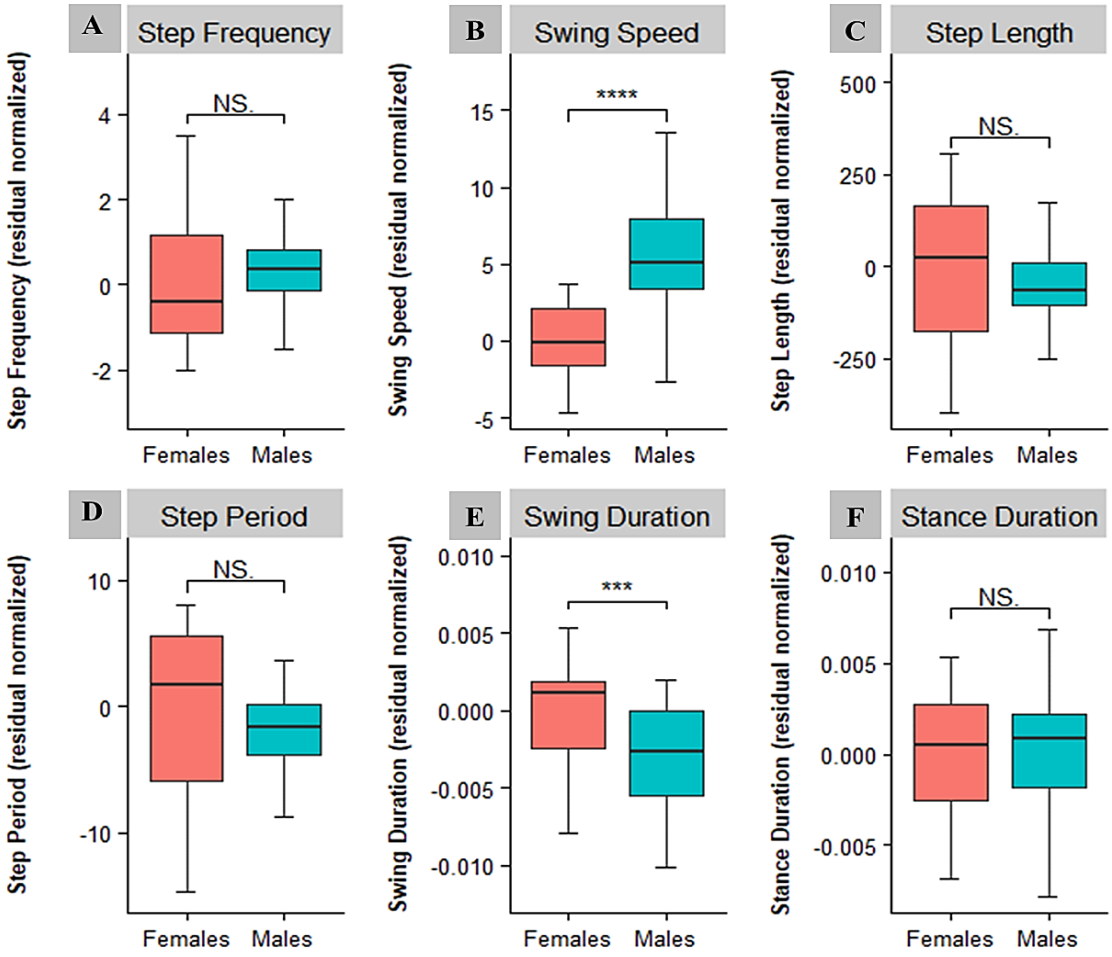

Figure 4.5. Boxplots' representation of differences between gender in general walking parameters. (A), (C), (D) and (F) In these specific parameters, the statistical differences are not significant, whilst in **(B) and (E)** the differences encountered are significantly different in both genders. Indeed, the increase of (B) swing speed in males causes the decrease of (E) swing duration. The median as the middle line, with the lower and upper edges of the boxes representing the 25% and 75% quartiles, respectively; the whiskers represent the range of the full data set, excluding outliers. Data was residual normalized and expressed as the difference to the control. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, * p-value <0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001.

In relation to the spatial parameters, there are significant differences in most of them, except in PEP footprint clustering. Contrary to what was expected, the previous conclusions about the PEP Footprint Clustering were not considered significantly important, but in AEP case the differences were considered statistically relevant. In this case, the males present higher values compared with females. This indicates that during walking the females touch the ground always in the same region, forming a footprint cluster (see **Figure 4.6 A, B**). Furthermore, the footprint alignment is higher for males, indicating that the footprints are less aligned (see **Figure 4.6 E**). With these conclusions, it can be assumed that females had better inter- and intra-leg coordination. Besides that, the females presented higher values for stance straightness (or lower values for stance linearity), which indicates that the movement of the legs is less jittery in relation of the body (see **Figure 4.6 C, D**).

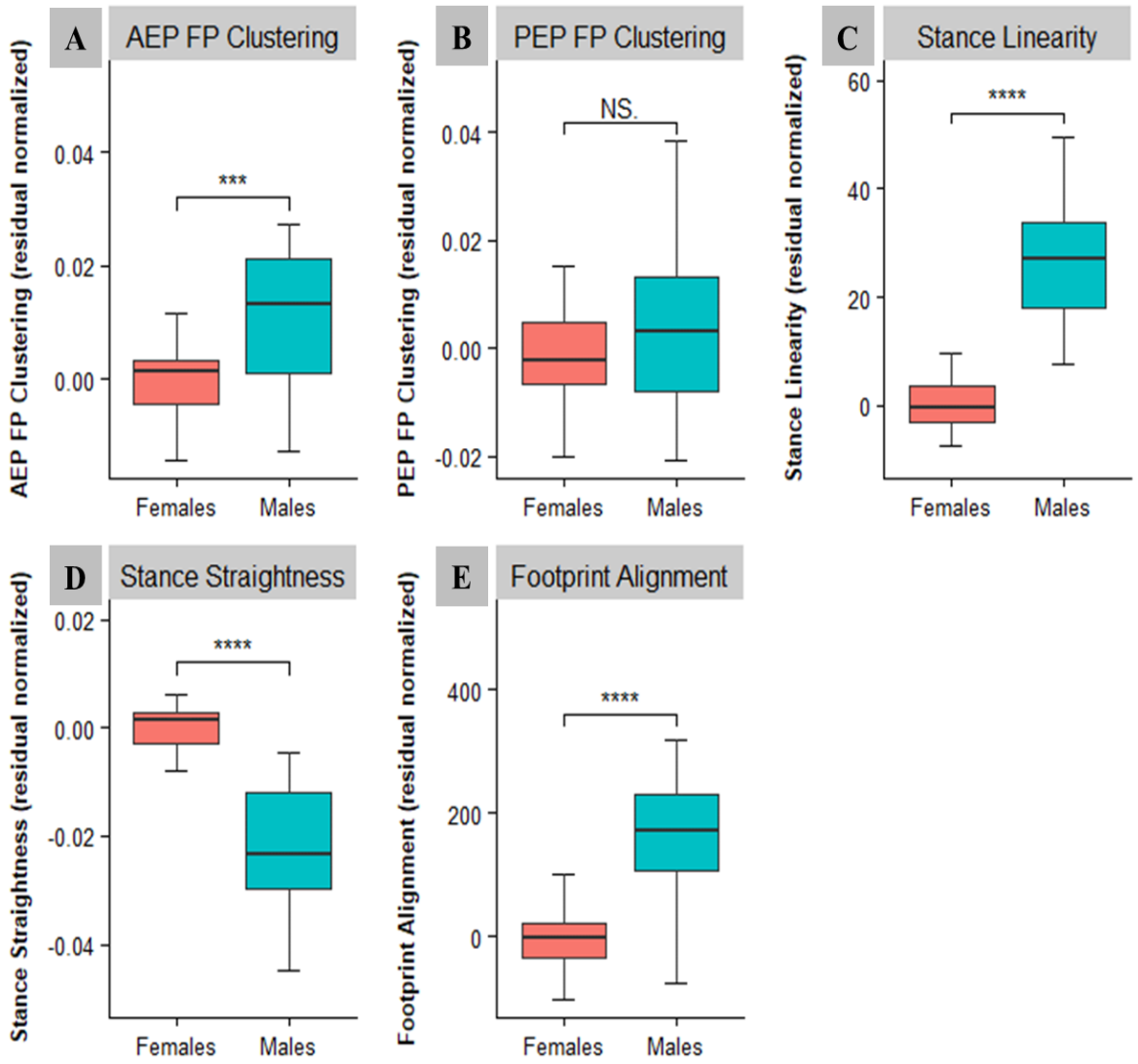

Figure 4.6. Boxplots' representation of differences between gender in spatial parameters. In the spatial parameters **(A), (C-E)** the differences are statistically significant. Only in **(B)** there are no differences between male and female flies, indicating that the PEP footprint position is similar in both genders. The median as the middle line, with the lower and upper edges of the boxes representing the 25% and 75% quartiles, respectively; the whiskers represent the range of the full data set, excluding outliers. Data was residual normalized and expressed as the difference to the control. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, *p-value<0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001.

For these measurements, it was plotted the AEPs (beginning of stance phase) and PEPs (beginning of swing phase) for each leg in relation to the center of the body (see Figure 4.7). Looking for the footfall positions, it seems that male flies occupy a larger area compared with female flies, indicated by the significant displacement in both directions (x and y directions). In other words, the tarsal contact of male flies tends to be further from the body center. Furthermore, the foreleg contact was shifted anteriorly, whilst the hind leg was shifted posteriorly, in both situations (AEP and PEP). Relative to the middle legs, the AEP position was shifted anteriorly, but the PEP position was shifted posteriorly.

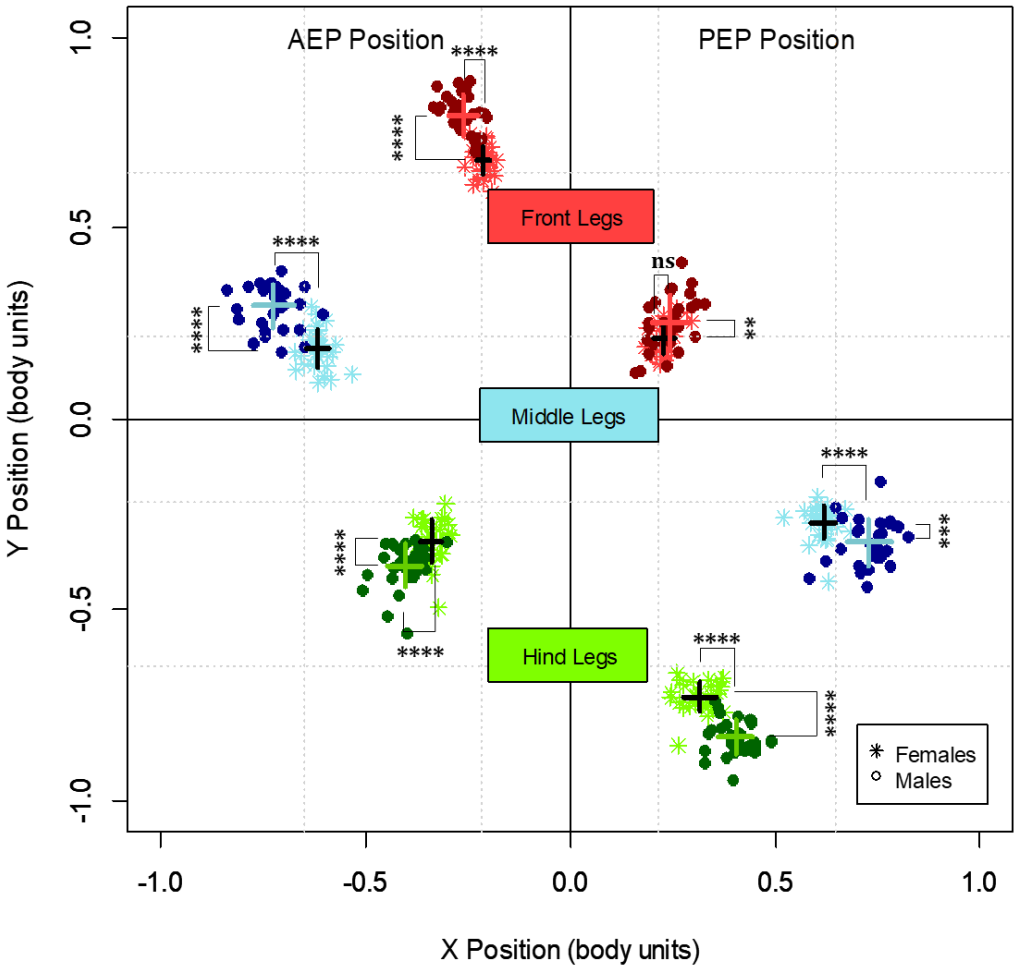

Figure 4.7. Representation of spreading of footfall positions relative to the body center (0,0) in males compared with female flies. The graph was divided into AEP and PEP footprint clustering, in the left and right side, respectively. The fulfilled circles represent all the footprints of males' stance position for front legs (in red), middle legs (in blue) and hind legs (in green); whilst, the stars represent the females' footprints with lighter colors. The size of lines in each cross denotes the standard deviations in each direction, whose intersection indicates the mean value of all footprints. Statistical analysis was determined using one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, *p-value<0.05; **p-value<0.01; ***p-value<0.001; ****p-value<0.0001. Each fulfilled circle or star result of one movie. In total were considered 30 movies from 10 different flies of both genders.

Normally, this spreading of tarsal contact is required in order to increase the static stability, maintaining the center of mass within the support area. So, according to this hypothesis, the spreading of tarsal contact in male flies may be due to the need to increase the static stability. However, contrary to what has been verified for the stance straightness parameter, the stability of male flies is lower.

Despite all these differences, both AEP and PEP shifted similarly, resulting in an unchanged step length (as demonstrated in **Figure 4.5 C**).

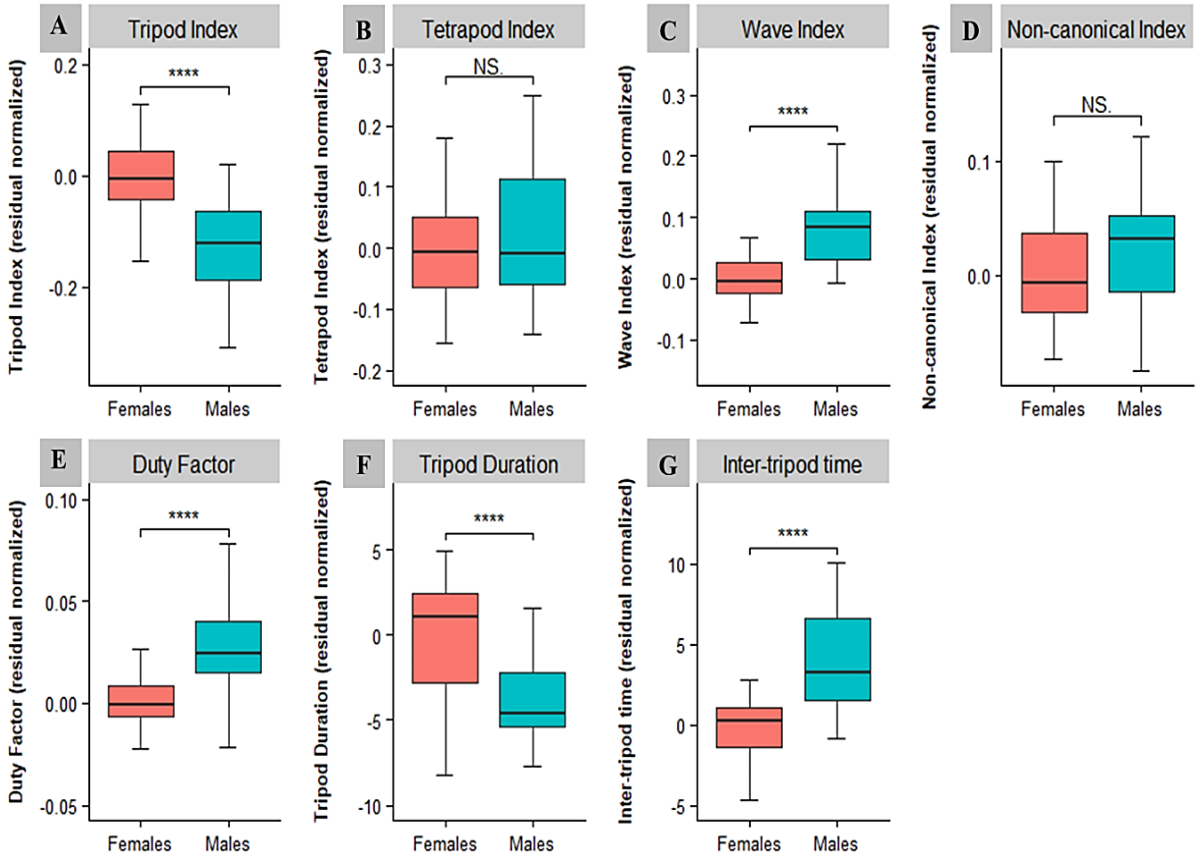

Figure 4.8. Boxplots' representation of differences between gender in gait parameters. Between both genders, there are no statistically significant differences in the (B) tetrapod and (D) non-canonical indexes. The median as the middle line, with the lower and upper edges of the boxes representing the 25% and 75% quartiles, respectively; the whiskers represent the range of the full data set, excluding outliers. Data was residual normalized and expressed as the difference to the control. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, *p-value<0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001.

According to the previous results, it confirmed that females use more tripod gait but less wave gait. In the other kind of gaits, there are no significant differences between both genders. Relatively with the duty factor, that is a measure used to distinguish walk from runs (see **Appendix A.3**) is significantly higher for males (see **Figure 4.8 E**). Although the duty factor is highly dependent on velocity, it was verified that regardless of speed, this parameter is a relevant factor in the distinction of walking in females and males. So, in 29% of females analyzed, the duty factor was lower than 0.5, indicating that some flies run instead of walking (see **Appendix A.3**). The inter-tripod time and the tripod time are closely related to the tripod index. Once the fly uses more tripod gait, as in case of females, the inter-tripod time is lower and the duration of usage this gait is higher. This can be also confirmed seeing the representative color codes in **Figure 4.4 C**, **E**. In the females, there are more regions colored with green than in males, which indicates that this tripod gait is preferable for females. But also, it is possible to see that the distance between each green region, the inter-tripod time is lower for females. And the area of each green region is higher for females.

4.1.2 Locomotion changes after amputation in both genders

Once the existence of relevant differences in walking behavior between unamputated female and male flies was observed, the next step is to quantify, also in both genders, the locomotion changes after debilitating motor conditions, like amputation and verify if there is motor adaptation and whether this adaptation can reach the normal walking pattern. With the amputation procedure, the normal sensory feedback is seriously compromised in two directions: from all distal leg segments to the lesion and from the lesion to the remaining legs. Besides this, the existent load sensors proximal to the lesion cannot provide information about the ground contact, causing a mechanically uncoupling relative to the remaining intact walking legs. To test the kinematic effect of that, it was used female and male flies (1-week-old) that suffered an amputation of both middle legs. The walking pattern of each fly was recorded using the *fTIR* assay 15 minutes, 1 hour, 24 hours, 72 hours and 7 days after amputation. For each time-point was filmed at least 20 movies (around 3 movies for each amputated fly). After the recording, the same analysis procedure was done. In this analysis, was verified that the first effect in the walking behavior is the decrease in the average speed. Once all the other parameters depend on the velocity, it is normal that this change is reflected in the remaining parameters. Consequently, it is expected that the step period suffers an increase, especially due the increasing of stance duration, but also due the slight decreasing of swing phase. This decrease can be reflected in the increasing of swing speed. Once swing speed increases, the initiation of the new stance period becomes faster and the step length shorter. Furthermore, it is expected that coordination decreases and the footprint clustering becomes higher (higher values represent less consistency of footprints). As a result, the movement of body center becomes wobblier and the stance straightness decreases. Furthermore, the inter-leg coordination becomes compromised because initially, the flies have 6 legs, but after the amputation procedure only remains 4 legs. With the only remains legs, the flies try to improve its gait, assuming new kind of gaits (*e.g.* pace, trot and walk gait) in order to maintain the inter-leg coordination.

To confirm these expectations, it was performed the residual analysis for each time point (considering the unamputated group as a control) and for both genders, in order to see whether flies were capable to improve its gait and reorganize their motor circuits to support the biomechanical challenges. Initially, it is possible to see some motor recovery by visualization of stance traces (its linearity indicates how much linear was the body movement relative to each footprint). With the amputation, the stance traces became wigglier compared with unamputated control, but over the time, despite the straightness values cannot reach the control values, the stance straightness suffers a significant increase 72 hours and 7 days after (see **Figure 4.9 A** and **C**). Besides this, it was observed that the amputation procedure affects the intra-leg coordination, increasing the footprint clustering, but only significantly in the AEP position. These conclusions are evident for both genders, but in males, there are no significant differences in the AEP footprint clustering over all the time points considered, indicating that amputation doesn't introduce any significant change in this parameter, remaining stable during the recovery process. Even so, it is evident that females can partially recover just after 1 hour, whilst males only can recover from 24 hours after the amputation (see **Figure 4.9 A**). Furthermore, 7 days after the amputation, the AEP footprint clustering in females almost reaches to the control values. In other words, there are no significant differences between unamputated flies and the 7 days amputated flies. So, it is possible to consider that there is a delay in males' recovery when it is compared with females.

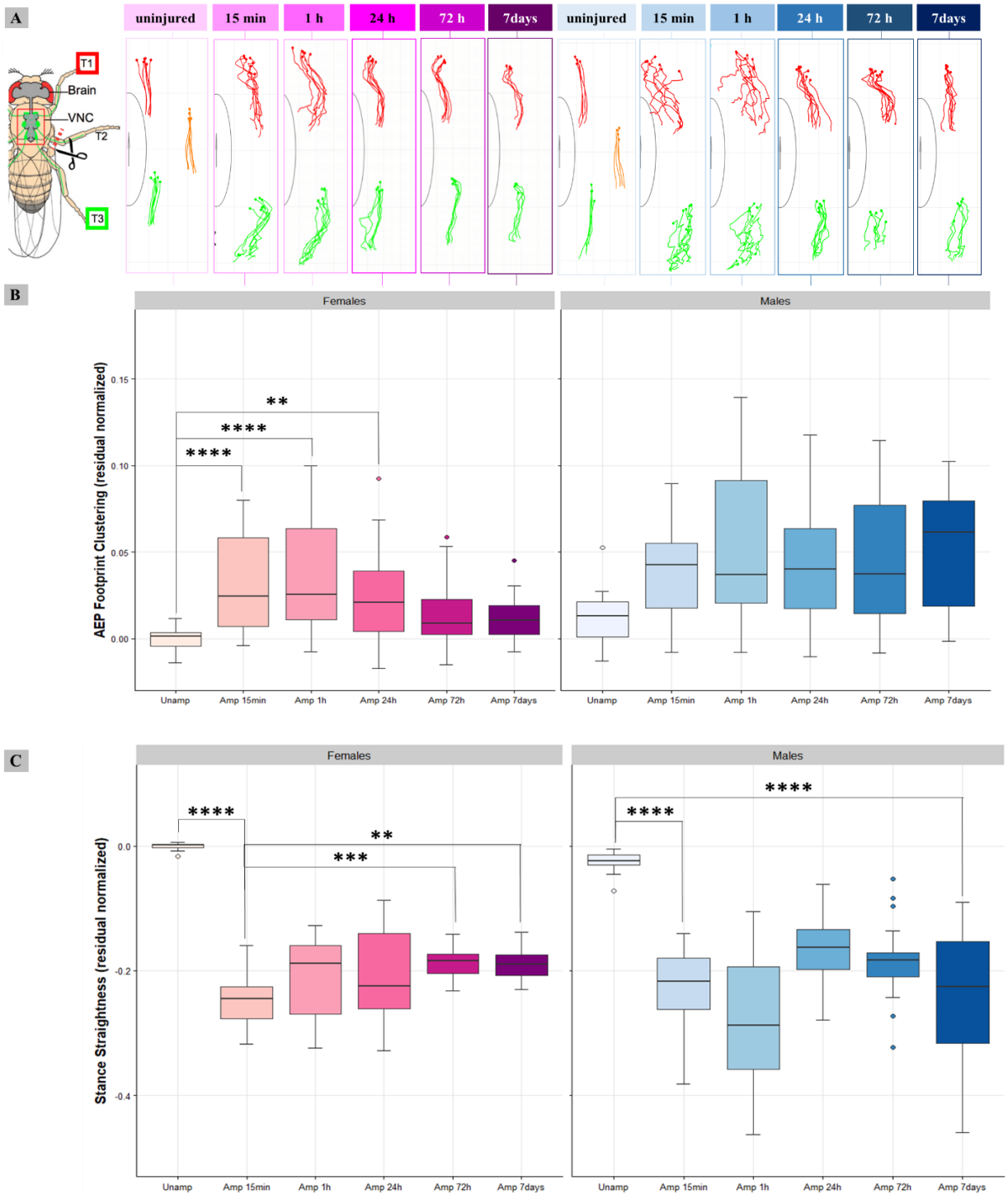

Figure 4.9. Comparison of evolution of motor recovery 15 minutes, 1 hour, 24 hours, 3 days and 7 days after amputation between female and male flies. (A) Stance traces representation of motor recovery, in which the Footprint Clustering and the Stance Straightness can be observed, over the five time points considered**.** The quantification of these two metrics was made in **(B) and (C)** boxplots, respectively. The median as the middle line, with the lower and upper edges of the boxes representing the 25% and 75% quartiles, respectively; the whiskers represent the range of the full data set, excluding outliers. Circles indicate outliers. Data was residual normalized and expressed as the difference to the control. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, *p-value<0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001.

In order to understand the footprint clustering results in both genders, it was represented the footfall position graph (see **Figure 4.10**). Here, it was possible to compare the evolution of motor recovery in

this specific parameter. According to these quantifications and the previous results relative to the kinematic comparison between genders, it was seen that there was a significant difference in the occupied area by footprints during the stance position. Despite the small size and the weight, the males seem to occupy a larger area during walking, represented by the displacement in both directions of AEP and PEP (see **Figure 4.10 B**). However, during the motor recovery, the AEP and PEP also seem to suffer an outer displacement, maybe in order to acquire more stability due to the inexistence of the two-supportive middle legs. But during the recovery process, in female flies, the footfall positions tend to approximate to the control values, especially in the hind legs (see **Figure 4.10 A**). Whilst in males, the footfall positions seem to progressively become more external, except in the AEP position of the front legs. Once more, it is possible to hypothesize that there is a delay in motor recovery of male flies. And the regression of footfall positions to the control values could occur at a further time point not quantified.

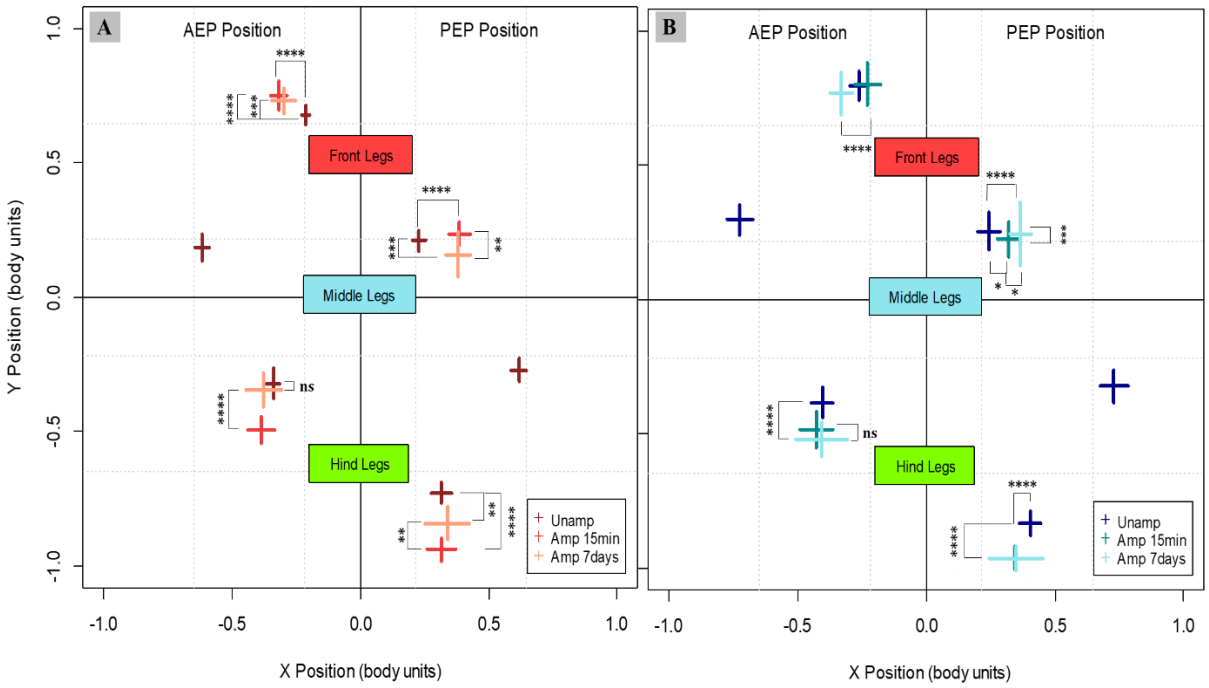

Figure 4.10. Comparison of evolution of footprint clustering recovery before and after amputation for (A) female and (B) male flies. The graph was divided into AEP and PEP footprint clustering, in the left and right side, respectively. The size of lines in each cross denotes the standard deviations in each direction, whose intersection indicates the mean value of all footprints. Statistical analysis was determined using one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, *p-value<0.05; **p-value<0.01; ***p-value<0.001; ****p-value<0.0001.

Like it was said previously, the amputation procedure induces immediate changes in gait, especially in step period. Normally, the amputated flies perform fewer step cycles during the same period, compared with healthy flies (see **Figure 4.11 A**, **B**). The amputated flies need to pass more time with the legs on the ground, in order to maintain the static stability. For these reasons, it is expected that the stance duration of amputated flies increased. And, as a consequence, the step period follows the same trend, whilst the swing duration tends to maintain constant. Indeed, the quantification of step period demonstrates that immediately after the amputation, the female flies increased their step period and over the recovery, it tends to stabilize. However, in male flies, the recovery process seems to be different. First, the amputation procedure immediately induces an increase of the step period for higher values compared with females, until 1 hour after amputation. Secondly, 24 hours after, the step period suffer

an abrupt decrease. Lastly, it suffers an another slight, but not significant, increase and then stabilizes (there are no significant differences between 3 and 7 days).

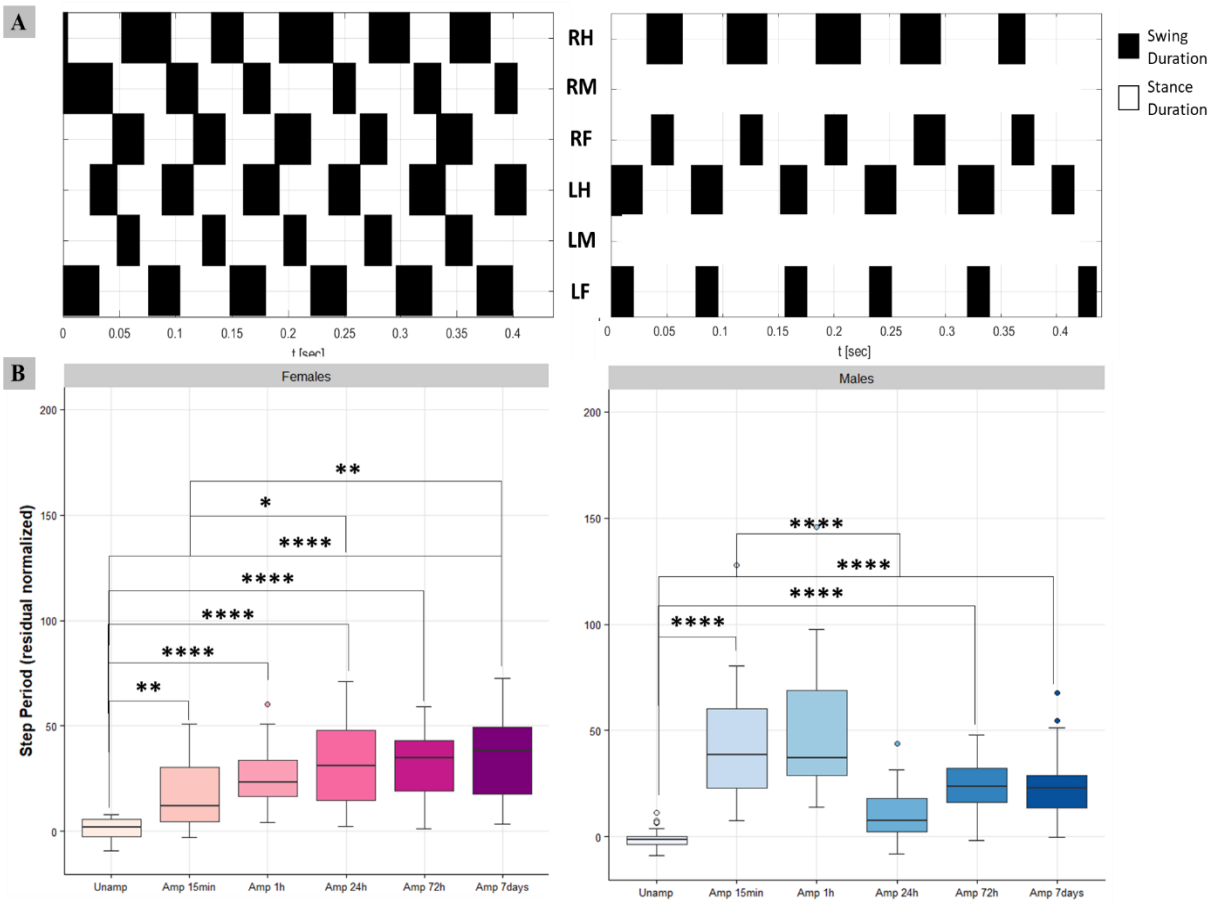

Figure 4.11. (A) Comparison of step pattern of the unamputated fly with the step pattern of injured fly in both middle legs, to see the immediate effect of amputation. Representation of kinematic changes promoted by the amputation: an increase of stance duration and the slight reduction of stance duration. Legend: RH – right hind, RM – right middle, RF – right front legs; LH – left hind, LM – left middle and LF – left front legs. **(B) Boxplots' representation of differences between gender in gait parameters, such as step period.** The median as the middle line, with the lower and upper edges of the boxes representing the 25% and 75% quartiles, respectively; the whiskers represent the range of the full data set, excluding outliers. Data was residual normalized and expressed as the difference to the control. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, * p-value <0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001.

In order to see, whether the step period trend was due the increasing of stance duration, it was also quantified the evolution of stance and swing duration over the recovery process (see **Figure 4.12**). Indeed, the amputation induces in females a decrease in swing duration and an increase of stance duration. As a consequence, the step period also increased. And this trend is maintained over the recovery process, in which the stance duration and the step period had similar values and the changes in the swing duration are mostly irrelevant. However, in males, the situation is completely different. Firstly, the swing speed only assumes positive values, which it means that immediately after the amputation, the swing duration suffers a slight increase, that is maintained until 1 hour after the surgical procedure. From 24 hours after amputation, the swing duration starts to decrease reaching the control values. The same trend is seen for the stance duration and step period. And in contrast with female flies, these two parameters assumed higher values immediately after amputation that then suffer an abrupt decrease

almost reaching the control values. From 3 days after the amputation, the step period and stance duration seem to stabilize in lower values compared with females.

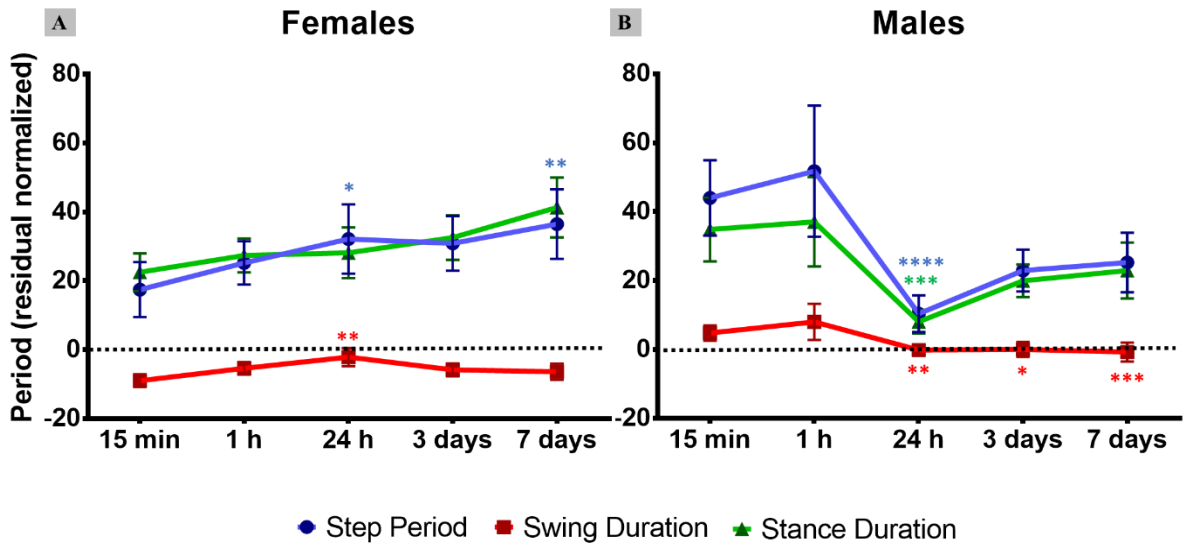

Figure 4.12. Representation of motor recovery over the course of few days relative to the step period, swing and stance duration for (A) female and (B) male flies. Each symbol indicates the mean value of residual normalized data with 95% confidence interval. Data was residual normalized and expressed as the difference to the control (represented by the dotted line in black and starting at zero) but the significant differences were represented relative to the 15 minutes' time point. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, *p-value<0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001.

The same procedure was made to step length and swing speed parameters (see **Figure 4.13 A and B**, respectively). Once again, in females, immediately after the amputation, the step length suffers an abrupt decrease that after seems to increase linearly with time of recovery but not significantly. In contrast, in males, the amputation induces an increase of step length, that after starts to decrease until 24 hours after amputation. From here, it suffers a slight and significant increase, that then is mostly maintained, despite the slight decrease. In swing speed, the trend is closely the same between both genders, except 7 days after the surgical procedure. Even so, the swing values from females are higher and the swing values from males are closer to the control values.

Besides the previously referred alterations, the adaptation of walking with only four legs passes by assuming new kind of gaits. These new kinds of gaits are characteristic of quadruped animals: pace, trot and walk gait (see **Figure 4.14**). Among these, the more stable is walk gait and the remaining are considered unstable because do not comply the static stability rule. For these reasons, 15 minutes after amputation, in the first contact with the new biomechanical challenges, the flies widely use the walk gait and some non-canonical gaits, in detriment of trot and pace gaits. Specifically, in females, 24 hours after the amputation the walk and trot indexes suffer a slight increase, promoted by the decreasing of pace and non-canonical indexes, although the absence of significant differences. In males, it has seen a similar trend. However, it is possible to denotate differences relative to the percentage of usage of each kind of gait. In male flies, the percentage of usage of walk and non-canonical gaits are lower than females; but, in contrast, trot and pace gaits are higher than females. In summary, the male flies tend to demonstrate a preferable use of unstable gaits (trot and pace); whilst, the female flies use mostly stable gaits and, maybe for this reason the stance straightness assumes a crescent curve of motor recovery in females (see **Figure 4.9 A**). In other words, the higher usage of stable gaits guarantees that female flies present a more coordinated walking, represented by stance traces with an increased linearity during the

recovery process. Contrariwise, in males, the stance straightness oscillation may be due to the preference of use unstable gaits. But it is also possible to hypothesize that this preference that causes the reduction of coordination, could be reflected an increase of coordination of the stump movements, once it was verified that is not mediated by a mechanical coupling of the legs when in contact with the surface.

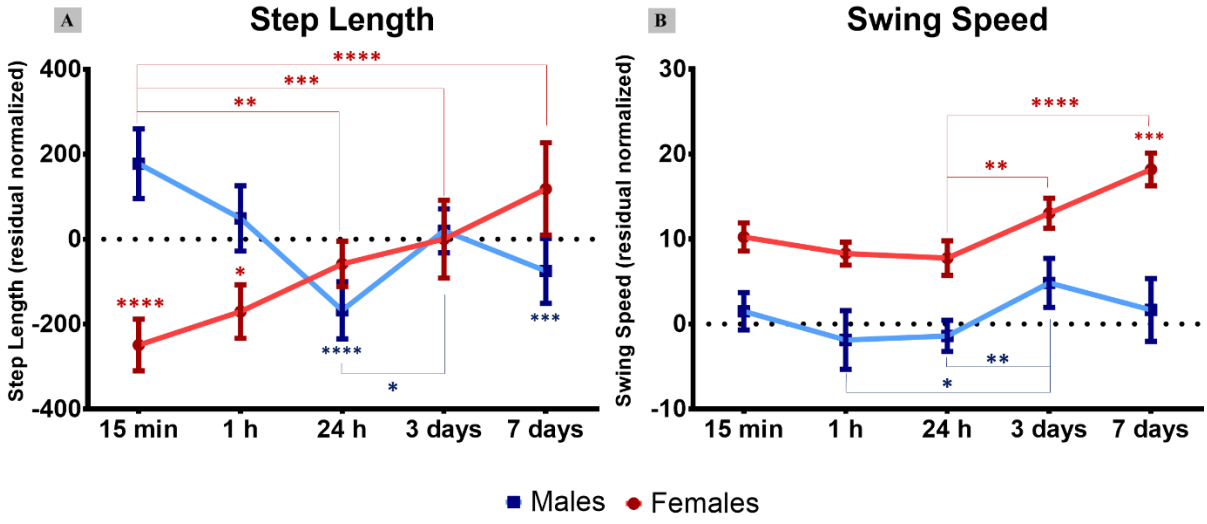

Figure 4.13. Representation of motor recovery over the course of few days relative to the step length and swing speed for (A) female and (B) male flies. Each symbol indicates the mean value of residual normalized data with 95% confidence interval. Data was residual normalized and expressed as the difference to the control (represented by the dotted line in black and starting at zero) but the significant differences close to the standard deviations were represented relative to the 15 minutes' time point. And the other significant differences are relative to the comparison between each time-point. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, *p-value<0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001.

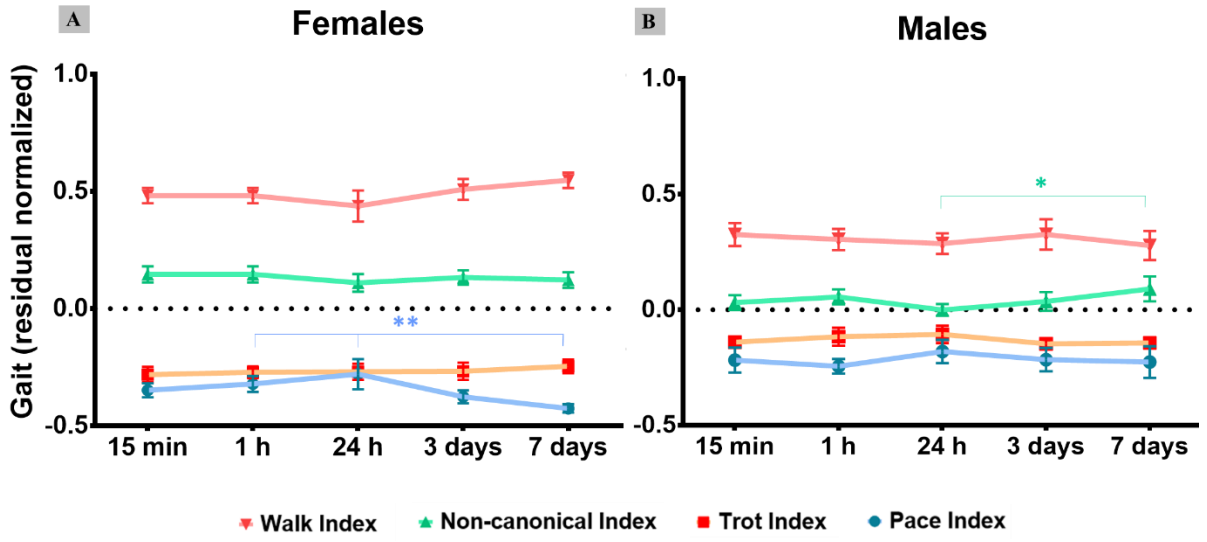

Figure 4.14. Representation of motor recovery over the course of few days relative each kind of gait for (A) female and (B) male flies. Each symbol indicates the mean value of residual normalized data with 95% confidence interval. Data was residual normalized and expressed as the difference to the control (represented by the dotted line in black and starting at zero). Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, *p-value<0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001.

In summary, these results demonstrated the intrinsic differences in the normal walking pattern between male and female flies. Based on these differences, it was expected that motor recovery process was also different. Even so, in some walking parameters, the motor recovery was seen in both genders. However, it was demonstrated that female flies had a motor recovery more evident and less oscillatory comparatively with male flies. For this reason, in the further goals were only used female flies.

4.2 Locomotor Recovery: age effect

In the previous results, it was demonstrated that gender was different effect in the recovery process after motor injury. Another physiological factor that seems to affect the motor recovery is the aging. For this purpose, it was tested only female flies with different ages. The ages were chosen in order to cover the full range of lifespan, that it is around 60 days (nearly 8 weeks). From here, the recovery process was tested in flies with 1, 3, 5 and 7-weeks-old using the same *fTIR* approach. Firstly, it was tested the normal locomotion of around 30 flies from different ages. And then, the amputation procedure was applied in at least 10 flies for each time-point considered and for each age. However, some limitations in terms of percentage of survival appeared, especially in flies with 7-weeks-old. In this stage, the percentage of survival is only around 20% and for this reason, the amputated flies barely survive, especially 7-days after the amputation (see **Appendix A.4**). Based on this, the recovery process it was only demonstrated over 2 time-points (15 minutes and 3 days after amputation).

Like it was said previously, the first step was to characterize the normal locomotion of flies with different ages and before that, it was considered some expectations about the effect of aging in walking parameters. According to these expectations, it was hypothesized that aging could induce a decrease of walking speed, an increase of stance duration and, consequently an increase of step period. As a result of a decrease in walking speed, the flies could increase the step frequency accompanied by a decrease of step length. Further, some of these expectations were confirmed and the first one was the walking speed. As expected, with aging, the flies tend to decrease the speed, consistent with observations in human patients (see **Figure 4.15 A**). However, for flies with 3-weeks-old occurred a totally unexpected abrupt increase of walking speed. From 1-week old to 3-weeks-old, the average speed increases around 70% (that is, from 33 mm/s to 48 mm/s). Further, it suffers a decrease for values lower than control (around 26 mm/s) and, so on. From 5-weeks-old to 7-weeks-old, the speed only suffered a slight decrease. The flies with 7-weeks-old walked with an average speed around 20 mm/s. Consistent with these results, flies with 3-weeks-old perform fewer step cycles, indicated by the lower value of the step frequency and over the course of weeks, the step frequency increased until reach the control values for 7-weeks-old flies (see **Figure 4.15 C**). These results indicate that age doesn't change the number of step cycles performed by flies, except for flies with 3-weeks-old. The same trend was seen in swing speed, however, in an inversely way. So, during the walking, the speed of legs during the swing phase do not suffer any significant change. In the same way, the step period is only significantly different for 3-weeks-old flies. Once more, the aging doesn't cause changes in terms of step period, stance and swing duration (see **Figure 4.15 B**). Consistent with the preliminary expectations, the step length suffers a significant decrease, except once more for 3-weeks-old flies. 3-weeks after, the decreasing of the distance between two consecutive steps is significantly higher, reaching the control values. From 5-weeks, it suffers another slight decrease to lower values (see **Figure 4.15 D**). These results indicate that older flies perform the walking behavior with lower step length.

Consistently, it was verified an abrupt increase in flies with 3-weeks-old for most of the walking parameters. However, this increase was not verified in the stance straightness parameter. Like it was said previously, this measure how much wobbly it is the walking movement of the legs relative to the center of the body. And according to this parameter, the wobbling increases with aging. It can be mean that with aging, the flies are less coordinated and have a less control of their balance during the walking (see **Figure 4.15 F**). Despite this observation, the walking coordination also quantified by the AEP and PEP footprint clustering increases (demonstrating that the footprints are less clustered) for flies with 3-weeks-old. It indicates that these flies have less control in the positioning of legs on the ground and over the weeks, it suffers a significant abrupt decrease. Although not significant, the abrupt decrease occurs for lower values than the control group (flies with 1-week-old), that are maintained until 7-weeks-old

(see **Figure 4.14 E**). So, with aging, the footprints became more clustered maybe, in order to acquire more stability during the movement.

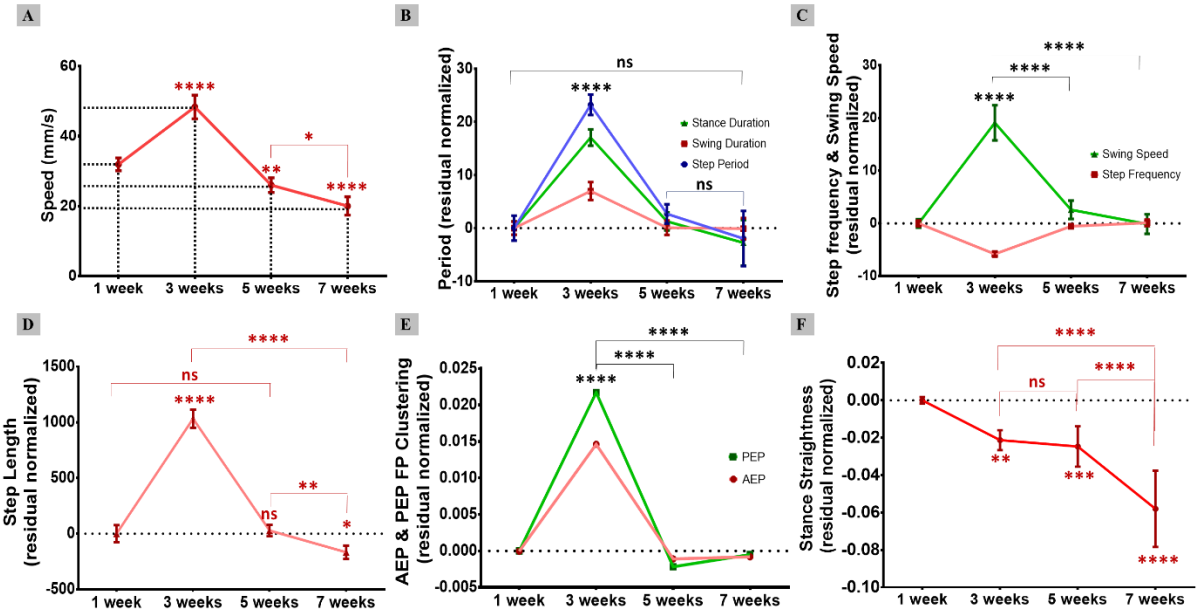

Figure 4.15. Representation of the effect of aging in healthy female flies walking kinematics. (A) – (F) The symbol indicates the mean value of residual normalized data with 95% confidence interval. Data was residual normalized and expressed as the difference to the control (represented by the dotted line in black and starting at zero). Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, *p-value<0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001. In (B), (C) and (E) the significant differences are represented in black because are common for all the variables represented in each graph.

In order to confirm the effect of aging in the footprint clustering were considered the footfall positions of all legs of each segment. From here, it was verified that aging induces a spread of tarsal contact. In other words, in the younger flies (with 1-week-old), the tarsal contact during the walking behavior was made close to the body. However, with the normal senescence, this trend changes and the tarsal contact is diffused from the body center. This spreading is more evident for 3-weeks-old flies. Despite this trend, it was verified that footfall positions of 1-week-old and 7-weeks-old flies are very similar (confirmed by the absence of significant differences between 1-week and 7-weeks-old in **Figure 4.15 E**). This may indicate that the motor learning process only starts after a week, suffering an optimization during the following 4-weeks. And from the 5-weeks, the motor learning seems to regress to the initial state. This regression is more evident in middle and hind legs (see **Figure 4.16**).

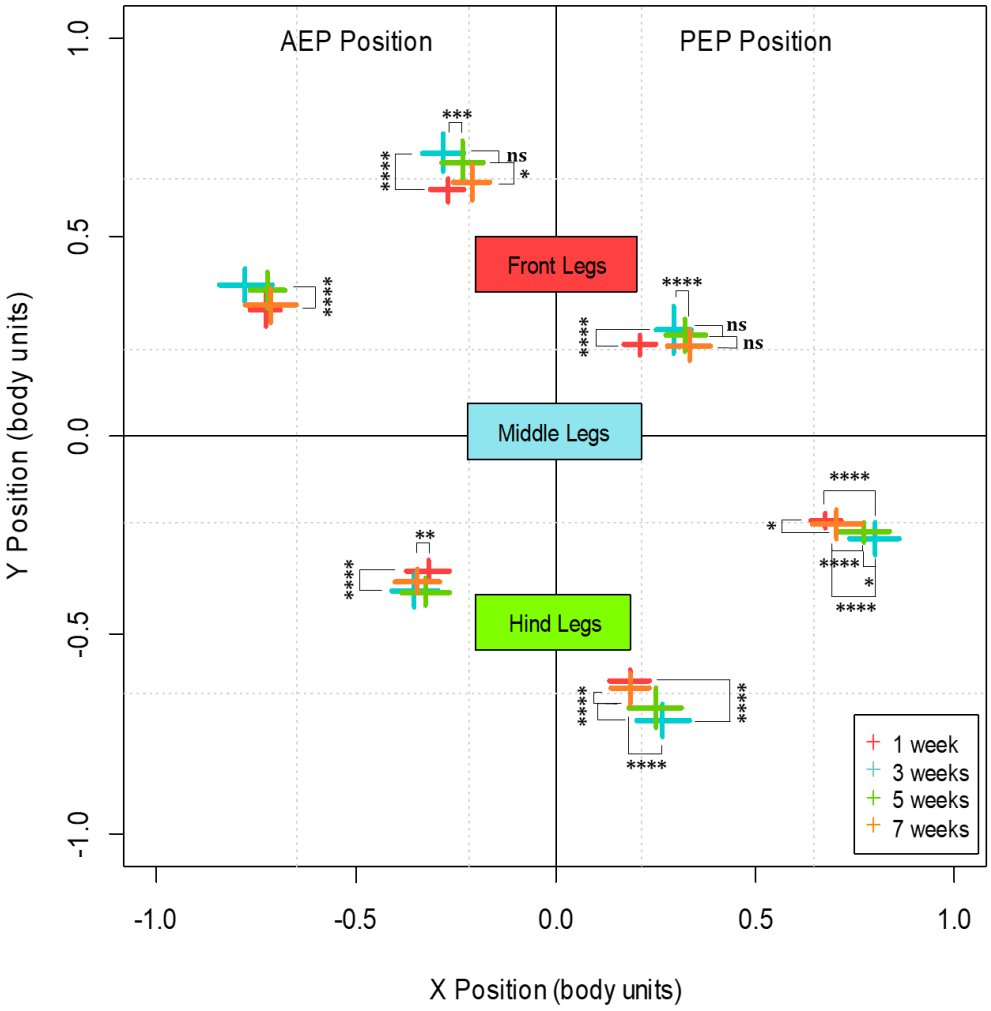

Figure 4.16. Comparison of footfall positions for healthy female flies with 1, 3, 5 and 7-weeks-old. The graph was divided into AEP and PEP footprint clustering, in the left and right side, respectively. The size of lines in each cross denotes the standard deviations in each direction, whose intersection indicates the mean value of all footprints. Statistical analysis was determined using one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, *p-value<0.05; **p-value<0.01; ***p-value<0.001; ****p-value<0.0001.

After this description, the amputation procedure was also applied in older flies in order to see whether aging is an intrinsic factor that affects the motor recovery after injury. As a preliminary expectation, it was considered that older flies cannot change their kinematic features to cope the loss of both middle legs. Despite this and similar with younger flies, the older flies would continue walking. For this purpose, the kinematic changes were quantified and the residual analysis was performed. From this analysis, it was possible to see that flies can still walk, but in a more uncoordinated way than younger flies. However, and according to the preliminary expectations, indeed there was a lack of motor recovery with aging. This lack of recovery is mostly seen in the stance traces representation (see **Figure 4.17 A**). Obviously, for 1-week female flies, the traces obtained 72 hours after the amputation are much more linear compared with the 15 minutes' time point, indicating a motor recovery. However, as the age advances, this motor adaptation is lost. In the further age stages, normally the traces obtained for 72 hours after the amputation seems to be worse than the traces obtained immediately after the surgical procedure. In other words, the traces became wigglier. Theoretically, it seems that with aging, the long-term memory is affected, whilst the short-memory is maintained. Despite this, it was seen that footprint clustering doesn't follow the same trend of stance straightness because the footprints seem to be more

clustered 72 hours after amputation in all age stages. These results are confirmed by the statistical quantifications' (see **Figure 4.16 B, C**).

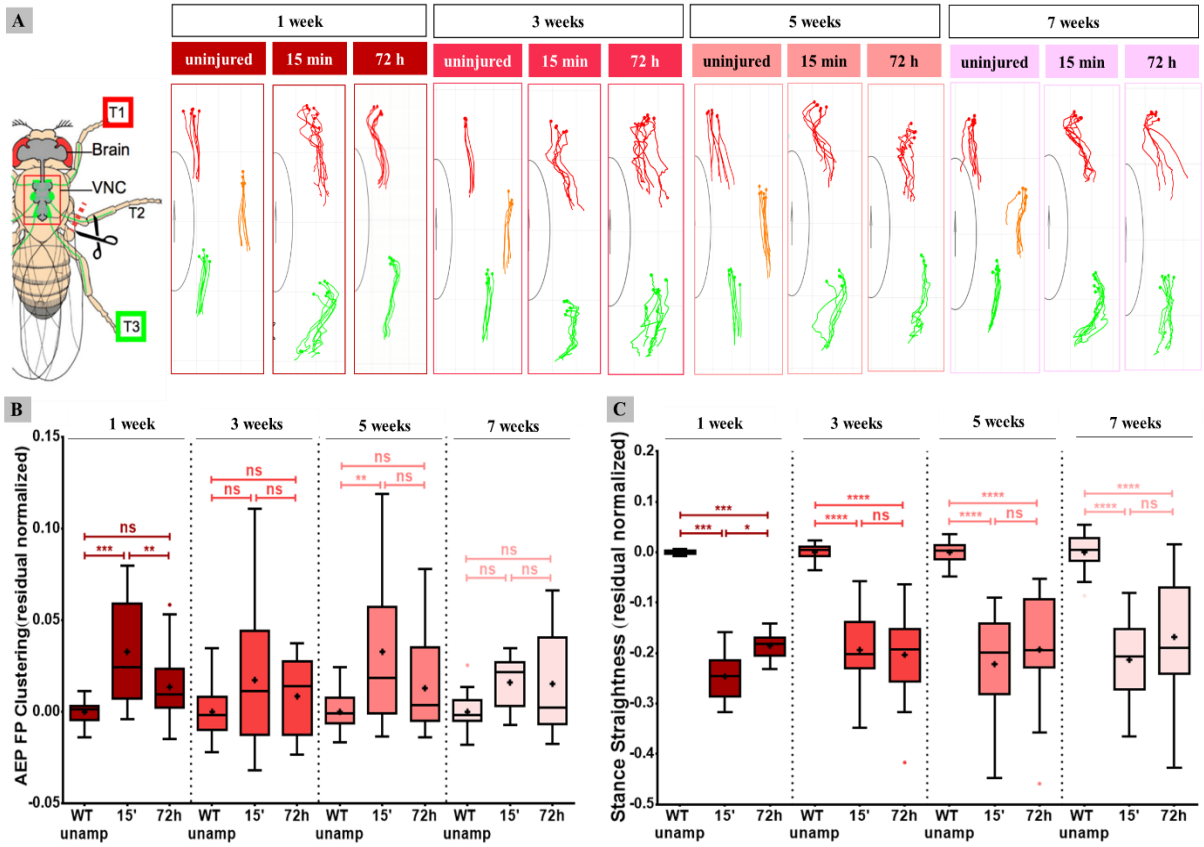

Figure 4.17. Representation of lack of motor recovery in older flies with 3, 5 and 7-weeks-old in the footprint clustering and in the stance straightness. (A) Evolution of stance traces over the weeks. The motor adaptation is only observed in the traces obtained for the 1-week-old flies. **(B), (C)** Quantification of footprint clustering (a measure of consistency of footprints for each leg) and the stance straightness (a measure of linearity of each stance trace). The median as the middle line, with the lower and upper edges of the boxes representing the 25% and 75% quartiles, respectively; the whiskers represent the range of the full data set, excluding outliers. Data was residual normalized and expressed as the difference to the control (1-week-old flies). Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, * p-value <0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001.

From these statistical quantifications', the motor recovery from the moment of amputation in 1-week-old flies is evident. When the flies were exposed to this new condition, the AEP footprint clustering suffers a significant increase, indicating that footprints became less clustered. In other words, the control of tarsal contact with the ground is less spatial restricted. But during the recovery period, the AEP footprint clustering decreases significantly, returning to the control values (there are no significant differences between the control and 72 hours' time point). It means that footprints became more clustered and the neural control of this return to the initial situation. This motor recovery was also seen in the stance straightness parameter. With amputation, the linearity of traces decreases, showing the loss of coordination and stability of movement. This reducing is quantified by a lower value of stance straightness (see **Figure 4.17 B**). With time, the same parameter suffers a slight increase, indicating the attempt to return to the initial condition before the amputation. However, this increase is not sufficient to reach the control values. For the further age stages, the recovery process is totally different. Although apparently, there is an increase of footprint clustering, in terms of statistical significance, there are no

differences between the two conditions (15 minutes and 72 hours). The abcense of significant differences can be masked by the higher dispersion of data (in other words, higher variability). The same trend is seen in the stance straightness. Another parameter that clearly demonstrates the motor recovery is the step length (see **Figure 4.18 A**). With amputation, the flies decrease the distance between two consecutive steps and during the recovery process, it suffers a significant increase to the control values. Relatively with 3-weeks-old flies, the amputation has a huge effect on the step length, taunting an abrupt decrease that is maintained over time. Contrary, in the other age stages, it seems that the amputation procedure doesn't have any effect because the amputated flies show similar values relative to healthy flies. As expected, the step frequency significantly decreases immediately after the amputation. And this decreasing is increased to 72 hours after the amputation (see **Figure 4.18 B**). This trend was also verified for the remaining age stages. And in all age stages, there are no differences between the two time-points considered after the amputation, except for flies with 7-weeks-old flies. Even so, the effect of aging seems to be higher for 5-weeks-old flies. To compensate this decrease, the younger flies increased significantly the swing speed after the amputation procedure (see **Figure 4.18 C**). Contrarily, in flies with 3-weeks-old, the amputation induces an immediate abrupt decrease, that after suffering an increase. In the remaining age stages, an increase over the recovery period it is verified. However, in flies with 5-weeks-old, there are no differences between the two time-points considered after the amputation. And in 7-weeks-old flies, the amputation once more did not have any effect on this parameter. Whilst, during the recovery, the swing speed suffer a significant increase.

Like it was said previously, the step period is the sum of the stance and the swing period. However, the swing period barely counts for this sum and the step period is mostly explained by the stance period. For this reason, the quantification of these two parameters is similar (see **Figure 4.18 D, E**). In both, it was verified that the amputation procedure induces a significant increase of stance duration and, consequently the step period. But, this increasing is only maintained over the recovery for the younger flies. Contrary to these results, the 3-weeks-old flies the amputation induce a significant decrease in the stance duration, that was not verified in the step period. In terms of swing duration, the amputation caused a significant decrease (see **Figure 4.18 F**). Over the recovery, it occurred a slight increase. In the remaining age stages, the amputation did not induce any change, but during the recovery, the swing duration suffered a decrease.

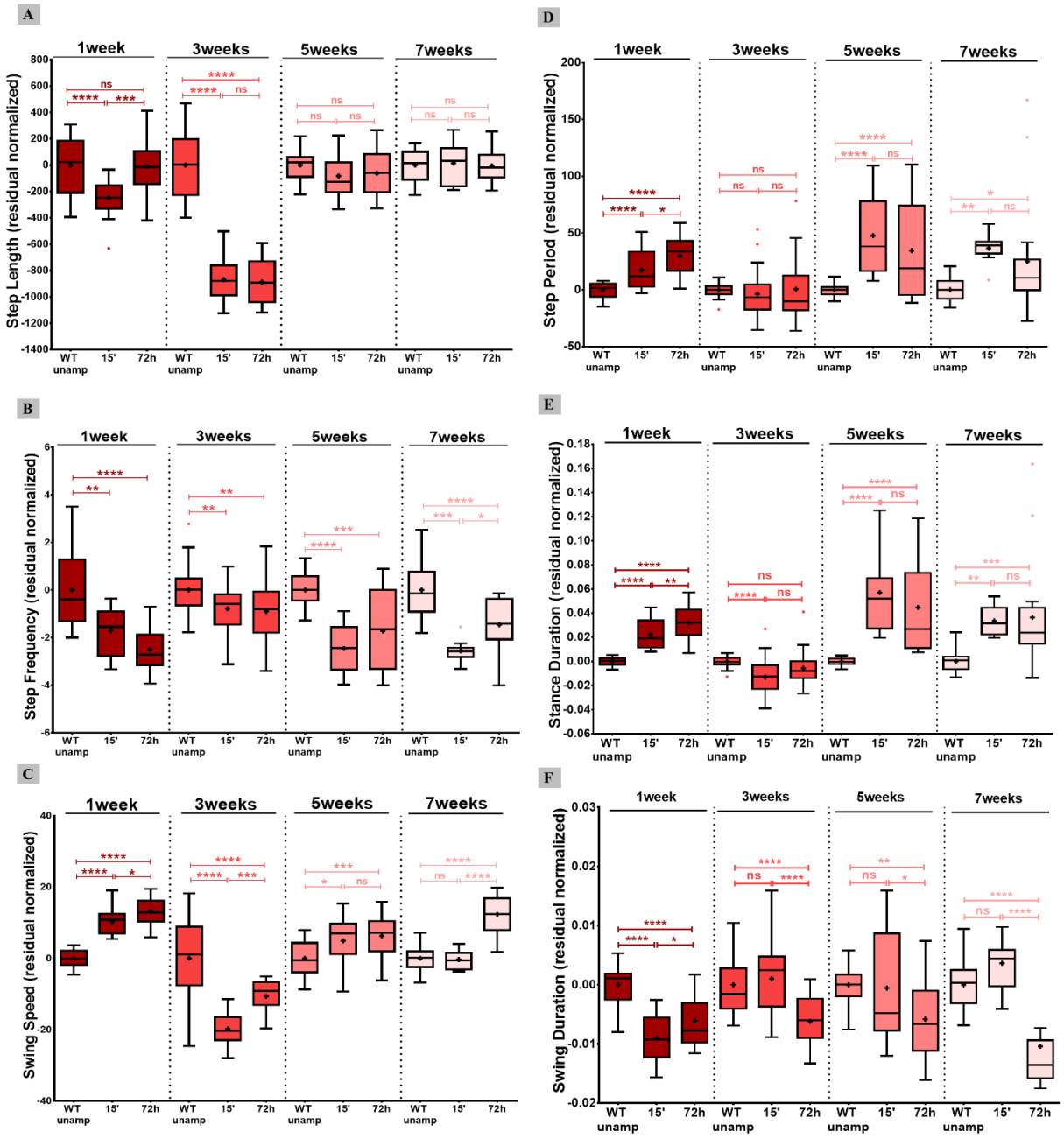

Figure 4.18. Representation of the lack of motor recovery in older flies with 3, 5 and 7-weeks-old in the remaining walking parameters. The median as the middle line, with the lower and upper edges of the boxes representing the 25% and 75% quartiles, respectively; the whiskers represent the range of the full data set, excluding outliers. Data was residual normalized and expressed as the difference to the control. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, * p-value <0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001.

In summary, these results demonstrated the effect of normal senescence in the normal walking pattern of female flies. According to these results, it was verified the kinematic changes caused by the aging. One of these kinematic changes was the reduction of average speed, starting at 5-weeks. Before this time-point, the average increases in an abrupt manner, especially for 3-weeks-old flies. In parallel, the remaining parameters (step length, step period, AEP and PEP footprint clustering and swing speed) also decreases from 3 to 7-weeks. Curiously, it was also demonstrated that the position of AEP and PEP of each leg changes differently. The most important evidence retired from these quantifications was the huge similarity between 1 and 7-weeks-old flies. Contrary to expectations, starting at 3-weeks the spread

of footprint relative to the center of body decreases. It indicates that from the 3-weeks the AEP and PEP positions tend to return to the positions of flies with 1-week. This returning was so evident that the positions obtained for 7-weeks is similar to the positions obtained for the 1-week-old flies, especially for middle and hind legs. This evidence may indicate that in 1-weeks-old flies, the level of learning acquired so far is equivalent to the level of unlearning in 7-weeks-old flies. However, after the amputation procedure, the 1-week-old flies, although the motor learning appears to be incomplete, they can recover from the amputation maybe due to the higher plasticity in remapping the uncompleted motor representations. While, in older flies (7-weeks-old), besides the lost the ability to learn, due to the normal senescence the motor plasticity is also reduced. For this reason, the ability to incorporate the new biomechanical challenges in order to remap the new motor representations is also lost. These hypotheses may be the justification of these kinematic changes, but remains the hypothesis for the major differences encountered for 3-weeks-old flies. Nevertheless, these kinematic changes with age can be explained by the exploratory activity.

4.3 Locomotor Recovery: activity effect

Over the years, it was already demonstrated that there is a progressive behavioral decline, especially senescence of motor activity with the normal aging. But remains to see the correlation between locomotor recovery and locomotor activity. In other words, it is necessary to test if flies that seems to recover from motor related injuries present a higher locomotor activity. And with these results demonstrate that older flies seem to have lack of motor recovery since they have less locomotor activity. For this purpose, it was tested only female flies with 1, 3, 5 and 7-weeks-old. Using the *FlyMotion* assay, it was possible to track the locomotor activity of 5 flies during 15 minutes at 20 frames/s. The quantification of each these group of 5 flies was made using the *FlyTracker* software. From here, x and y positions of each fly in the arena, among other parameters, were acquired in order to characterize the path of the total movement. Using the information about x, y position, it was possible to reconstruct individually the walking trajectory of each fly without losing the identity of each one. Just for simplicity, the further representations of the walking trajectory were obtained only using one fly that is representative of the data set (see **Figure 4.19**). According to these results, some differences between the unamputated controls for each age stage are visible. Over the weeks, the percentage of activity and the total distance traveled seem to be similar, except for 7-weeks-old flies. Even so, the 3-weeks-old flies assumed higher speeds over the 15 minutes considered, compared with 1 and 5-weeks-old flies. Besides this, the effect of amputation is clearly demonstrated. In all age stages, the amputation procedure induces an immediate decrease of locomotor activity. As expected the flies walk shorter distances during the same period considered for healthy flies. Another effect of amputation is the abrupt decrease of the instantaneous speed, represented by the red circles over the path (grey line). Once more, the motor recovery is visible. In other words, it is clear that flies can readjust their motor representation in order to adapt to the new biomechanical challenge. The adaptation allows flies walk increasing distances over the time. This adaptation is visible for 1, 3 and even for 5-weeks-old flies. However, for the 1-week-old flies, it seems that flies readjust their movement more effectively 24 hours after the amputation, instead of 72 hours after. Furthermore, in these last time-point, it seems that flies increase the percentage of centrophobism. In other words, the flies walking more in the periphery of the arena. In contrast, the 3-weeks-old flies, until 72 hours after the amputation, can recover in a gradual way. As a consequence, the total distance traveled and the percentage of activity increased also in a gradual way. Besides this, in the last time-point, the flies can also increase the instantaneous speed for values above the 20 mm/s (blue circles). So, it seems that that flies with 3-weeks-old can recover from motor related injuries more quickly than 1-week-old flies. In 5-weeks-old flies, the trend is similar. However, with advancing age, the flies practically walk only with an instantaneous speed below 4 mm/s (red circles). In the last age stage considered, in contrast to what was expected, 24 hours after the amputation adaptation is similar to the control. Nevertheless, the total activity and the total distance traveled seems to be lower than control. With the amputation, these flies barely move and this inactivity is also observed 72 hours after the amputation.

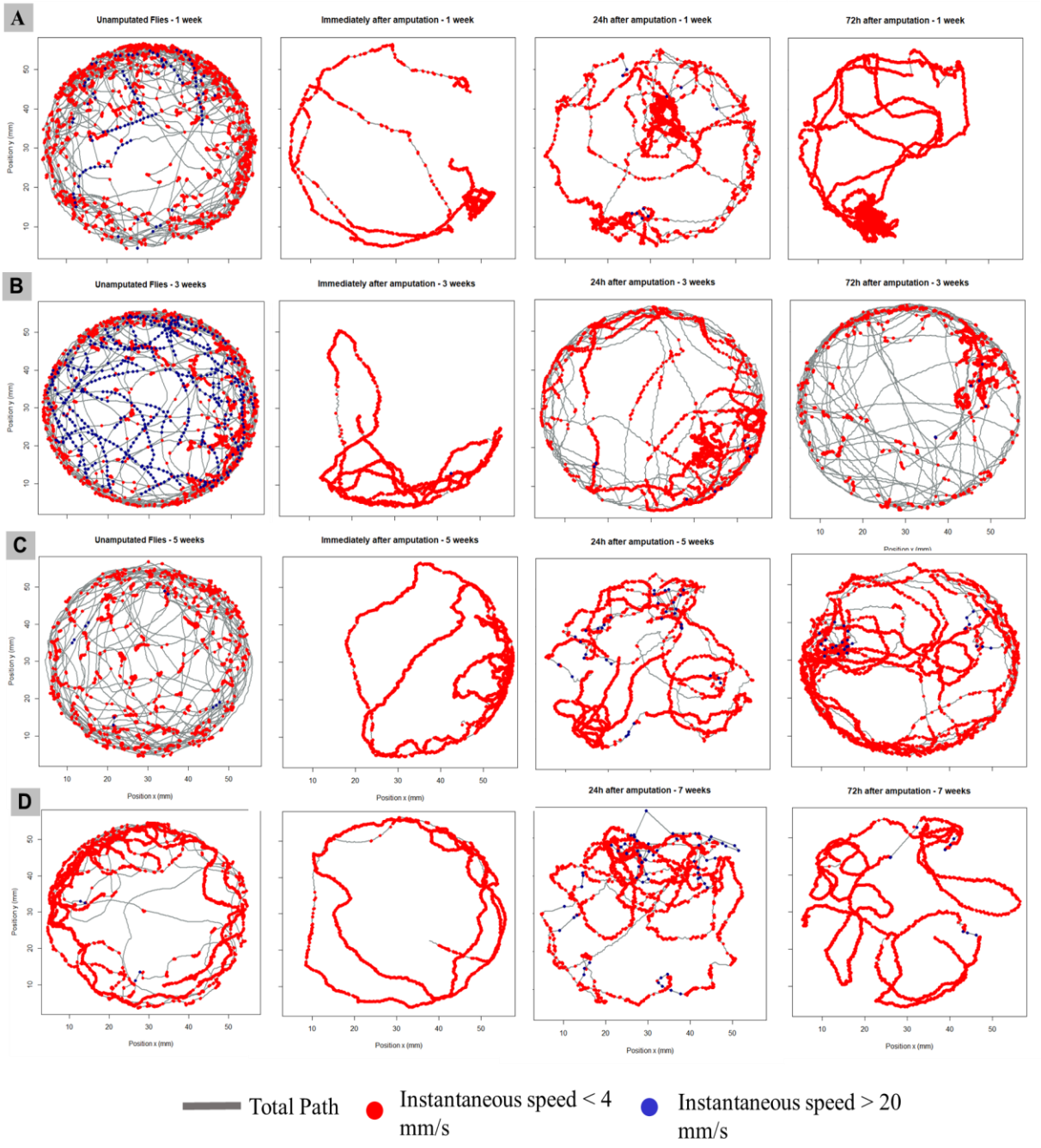

Figure 4.19. Evolution of total activity for flies with 1, 3, 5 and 7-weeks-old. Representation of the total path only for one representative fly, acquired over 15 minutes at 20 frames/s. In each representation, the total path was represented by a grey line. The red circles represented the instantaneous speed over the path below 4 mm/s, whilst the instantaneous speed above 20 mm/s are represented by blue circles.

Besides these qualitative quantifications, it was performed quantitative calculations. Some of these were obtained immediately after, from *FlyTracker* software, but other parameters were added posteriorly with a GUI made in MATLAB. Some of these new parameters added were the total distance, centrophobism and total activity (see Materials and Methods). From these, some previous conclusions were confirmed. Relative to the 1-week-old flies, it was verified that indeed the amputation induces an abrupt decrease of speed (see **Figure 4.20 A**). Over the days, it seems that there is a slight increase; however, this increase is not statistically significant. So, statistically, there are no differences between all the time points considered (15 minutes, 24 and 72 hours), indicating that the effect of amputation in

the average speed is maintained over all the recovery period. As previously verified, the percentage of centrophobism increases, reaching the control values (there are no significant differences between the control and the last time-point, 72 hours after the amputation) (see Figure **4.20 B**). However, this decrease in central exploratory behavior normally associated with an increase of anxiety (Ramos, 2008) is considered unexpected. The initial expectation was that recovered flies presented a higher exploratory activity in the inner region of the arena. And despite the inexistence of significant differences between the 72 hours' time point and the control group of flies, this last presented a similar percentage in the inner and outer regions, previously defined (see Materials and Methods).

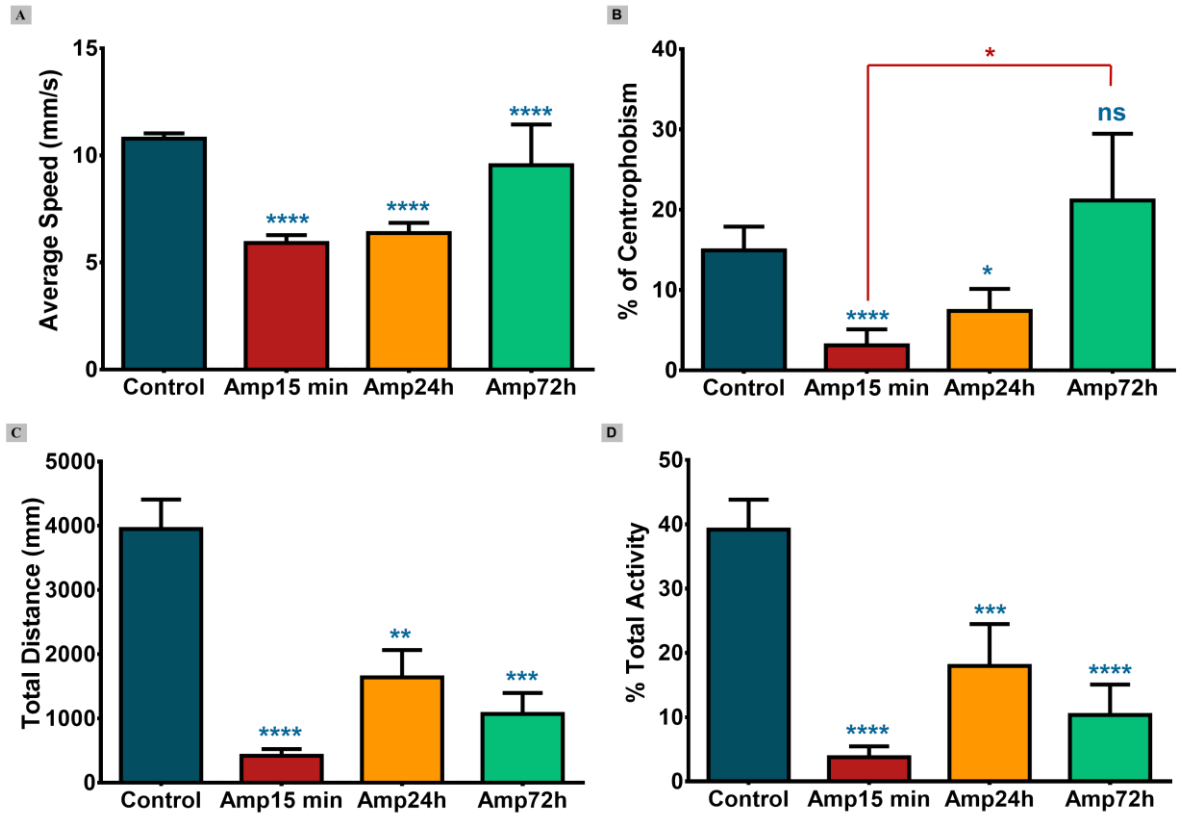

Figure 4.20. Evolution of activity parameters for flies with 1-week-old. In all the activity parameters is explicit the effect of amputation and the trend of motor adaptation during the recovery. The values shown are the mean ± SEM. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, * p-value <0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001. The significant differences in blue are relative to the control group of unamputated flies.

Relative to the remaining parameters (total distance and percentage of total activity), these are positively correlated (see **Appendix A.5**). It means that flies that walk greater distances have a higher percentage of activity, as expected. So, the trend seen in these two parameters is similar. With amputation both the total distance traveled and the percentage of total activity suffered an abrupt decrease that with the passage of a few hours, more specifically 24 hours, it suffered a slight increase, but not significant. And contrary the expectations, 72 hours after the amputation, back to decrease. Despite this, there are no significant differences between 24 hours and 72 hours' time points (see **Figure 4.20 C, D**), which means that despite the slight decrease from 72 hours after, this decrease is not significant, and the motor recovery presented 24 hours after is similar to the motor adjustment presented 72 hours after the amputation.

In the 3-weeks-old flies, the trend is similar, except for centrophobism. The big differences are in the total distance and in the percentage of total activity. In this case, the increase of both these two parameters is significantly higher, reaching the control values, 24 and 72 hours after the amputation (see **Figure 4.21 C, D**). In terms of speed, there is also a significant increase over the days, not existing differences between 24 and 72 hours after the amputation. There are no statistically significant differences in the centrophobism.

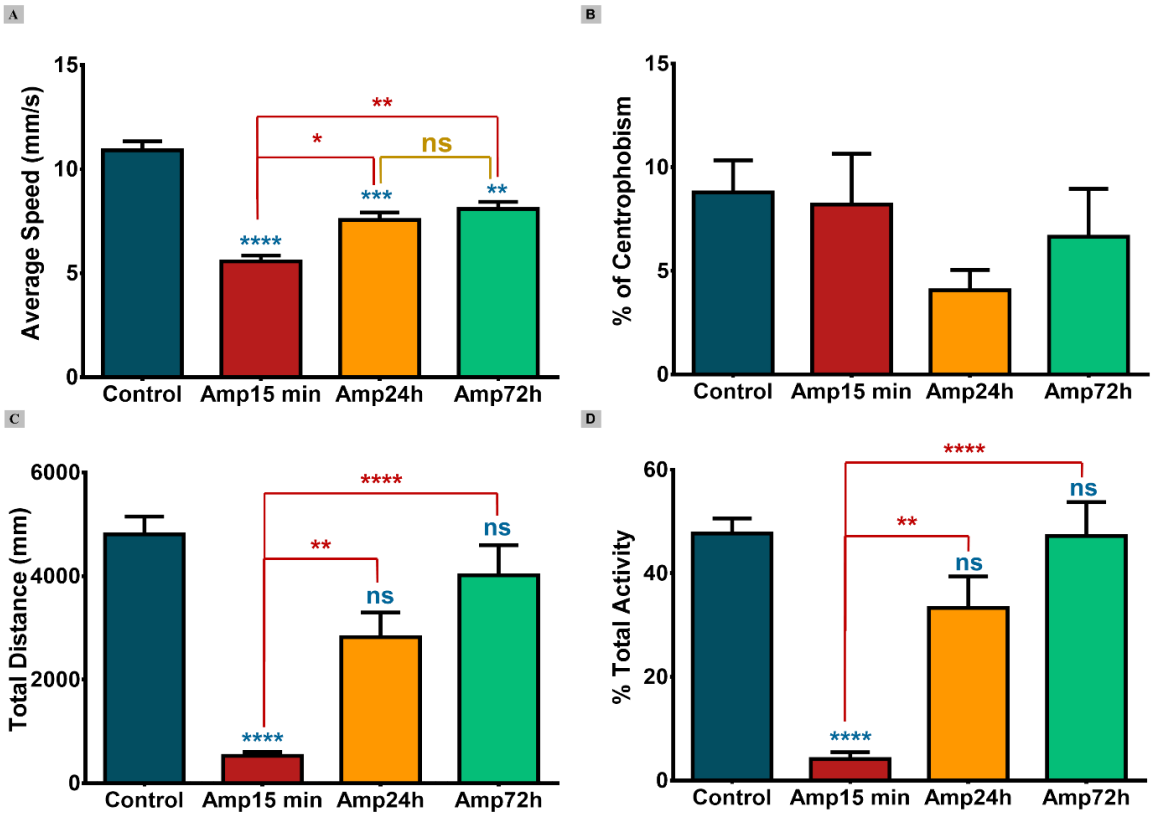

Figure 4.21. Evolution of activity parameters for flies with 3-weeks-old. The values shown are the mean ± SEM. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, * p-value <0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001. The significant differences in blue are relative to the control group of unamputated flies.

The average speed in 5-weeks-old flies abruptly decreases with the amputation, but 24 hours after the amputation the speed back to increase above the control values (see **Figure 4.22 A**). For this reason, there are no differences in the average speed between the unamputated control values and 24 hours' time-point. From here, the average speed back to decrease. Despite this, the inexistence of significant differences between the two-last time-points (24 and 72 hours after the amputation) indicates that average speed is practically maintained over the hours of recovery. In terms of centrophobism, there is a slight increase, however, non-significant (see **Figure 4.22 B**). The increase is only significant immediately and 24 hours after the amputation. In conclusion, there are no major changes in this parameter. Once more, the total distance and the percentage of total activity have a similar trend (see **Figure 4.22 C, D**). This trend is manifested by a gradual increase of both parameters, immediately after the amputation. The gradual increase is only significant relative to the control group, not existing differences between each time-point considered.

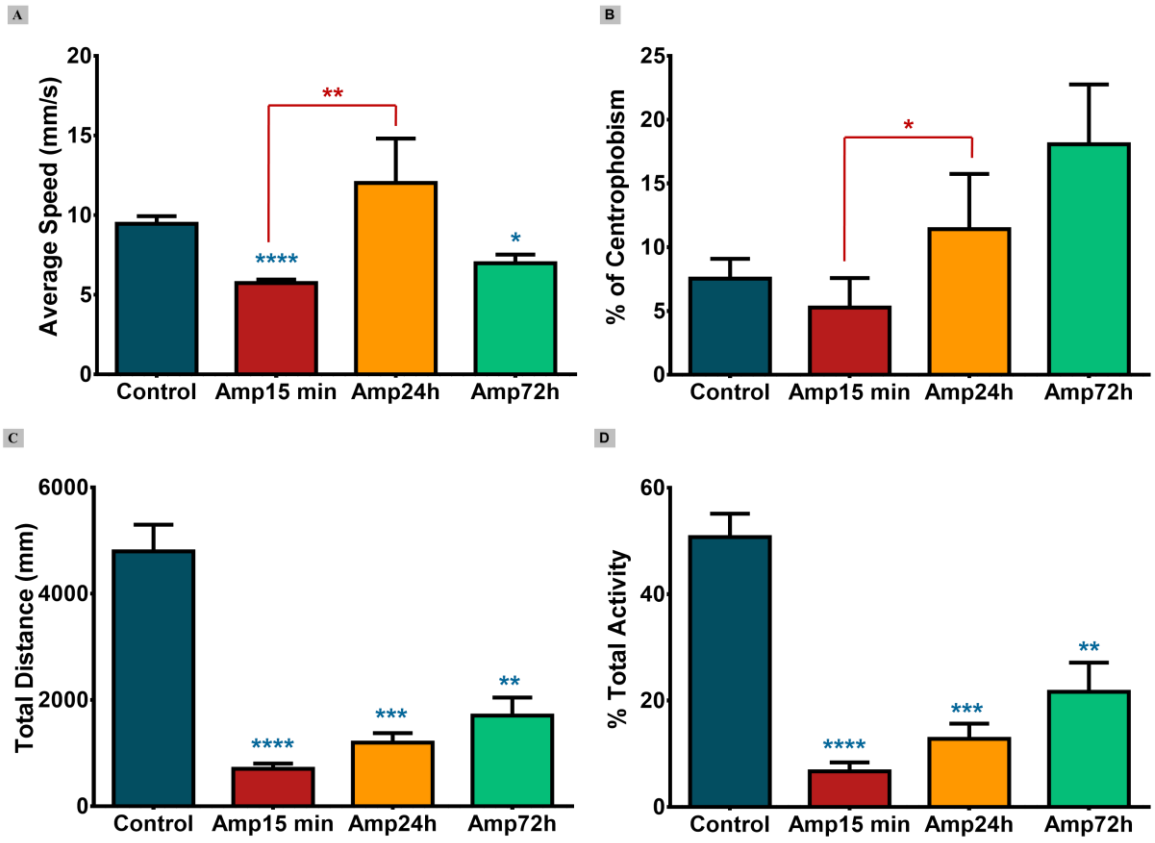

Figure 4.22. Evolution of activity parameters for flies with 5-weeks-old. The values shown are the mean ± SEM. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, * p-value <0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001. The significant differences in blue are relative to the control group of unamputated flies.

In contrast, the 7-weeks-old flies do not present any change in the average speed and in the centrophobism (see **Figure 4.23 A, B**). In this case, the amputation doesn't have any effect on the average speed and it is not evident any recovery. Similar to 3-weeks-old flies, 7-weeks-old flies do not show any significant difference in centrophobism. Despite this, it is seen a slight decrease from immediately after the amputation until 24 hours after the amputation. 72 hours after the amputation occurs an increase, but not significant. Furthermore, the amputation has an abrupt decrease of total distance and in the percentage of total activity (see **Figure 4.23 C, D**). However, 24 hours after the amputation, both parameters increase to control values (there are no significant differences between the control group and 24 hours' time-point) and then both parameters back to decrease, but it is only significant in the percentage of total activity.

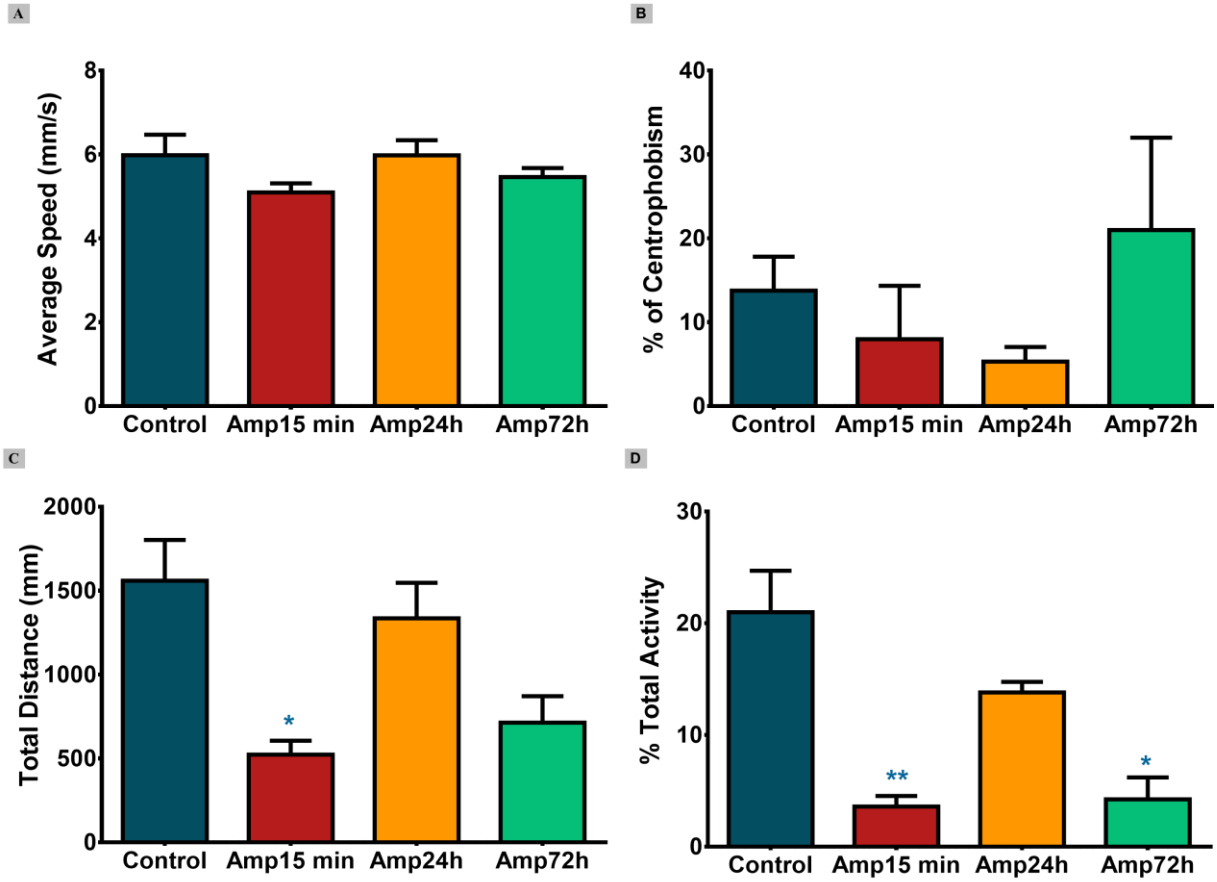

Figure 4.23. Evolution of activity parameters for flies with 7-weeks-old. The values shown are the mean ± SEM. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, * p-value <0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001. The significant differences in blue are relative to the control group of unamputated flies.

The significant differences between the controls and between each time-point considered were also observed. Comparing the control groups of flies, the most significant differences occurred between 3-weeks-old with the remaining age stages, except with 1-week-old flies. As expected, over the weeks, the average speed decreases, especially from 5 to 7 weeks after, there were no differences between 1 and 3-weeks. The effect of amputation in the average speed is similar over the weeks and the adaptation 24 hours after the amputation is also similar. Only in the 72 hours' time-point, there are differences over the weeks. And these differences are a slight increase of average speed from 1-week to 3-weeks and a gradual decrease from 3-weeks until 7-weeks (see **Figure 4.24 A**). In centrophobism there are no differences in all age stages. In terms of total distance and percentage of total activity, the trend is similar. Comparing the controls from 1-week to 5-weeks, the percentage of total activity and the total distance increase gradually, however in a statistically non-significant manner. From 5-weeks, both parameters suffer an abrupt decrease. In the following time-points (immediately and 24 hours after the amputation), both parameters assumed values similar over the weeks, indicating that amputation has the same effect in all age stages. Nevertheless, the effect is lower in 5-weeks-old flies. Whilst, in 24 hours' time-point, both parameters suffer a significant increase for 3-weeks-old flies. At the last time-point, contrary to the expected, the higher values of these both parameters were seen in flies with 3-weeks (represented by an abrupt and significant increase from 1 to 3-weeks). And from 3-weeks, both parameters decrease gradually, not existing differences between 1,5 and 7-weeks.

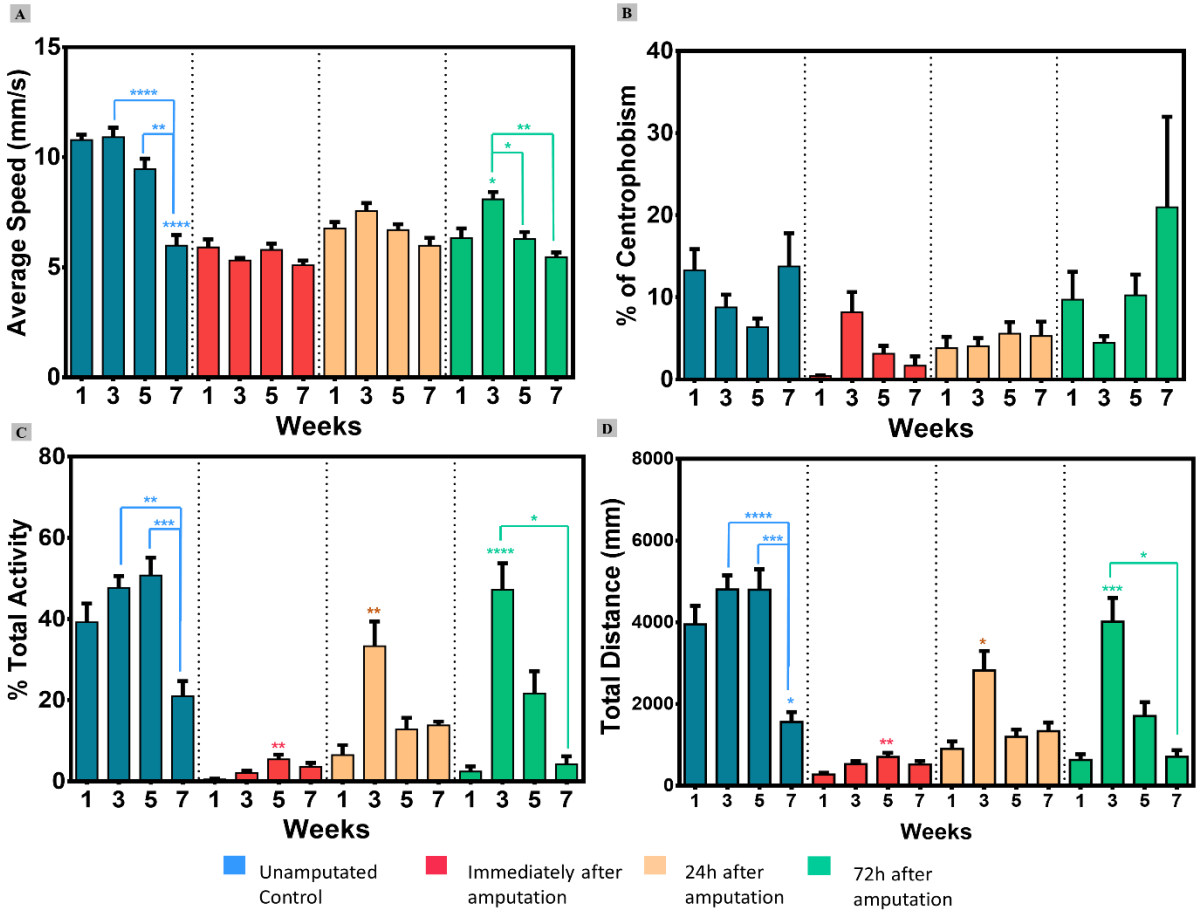

Figure 4.24 General panoramic of evolution of activity parameters over the weeks to control and each time-point considered. The values shown are the mean ± SEM. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, * p-value <0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001. The significant differences in blue are relative to the control group of 1-week flies.

Regarding the straightness of movement, it was studied the evolution of this parameter over the weeks. There is evidence that the straightness of movement decreased with the amputation, except for flies with 7-weeks-old. For these flies, once more, it was verified that injury did not affect the normal locomotion. For both 1 and 3-weeks-old flies, during the recovery period, the straightness of path increased, reaching the control values (there are no differences between the control group and 24 and 72 hours' time point). (see **Figure 4.25 A** and **B**). From 5 weeks-old, the motor recovery seems to be difficult. Upon injury, the straightness decreased significantly. During the motor recovery, it suffered a slight increase. In contrast to 1 and 3-weeks-old flies, the straightness of movement of 5-weeks-old flies did not reach the control values (see **Figure 4.25 C**). For 7-weeks-old flies, there are no significant differences (see **Figure 4.26 D**). This can suggest that locomotion features before and after injury are similar. Furthermore, it demonstrates that the effect of aging has the same effect of amputation procedure.

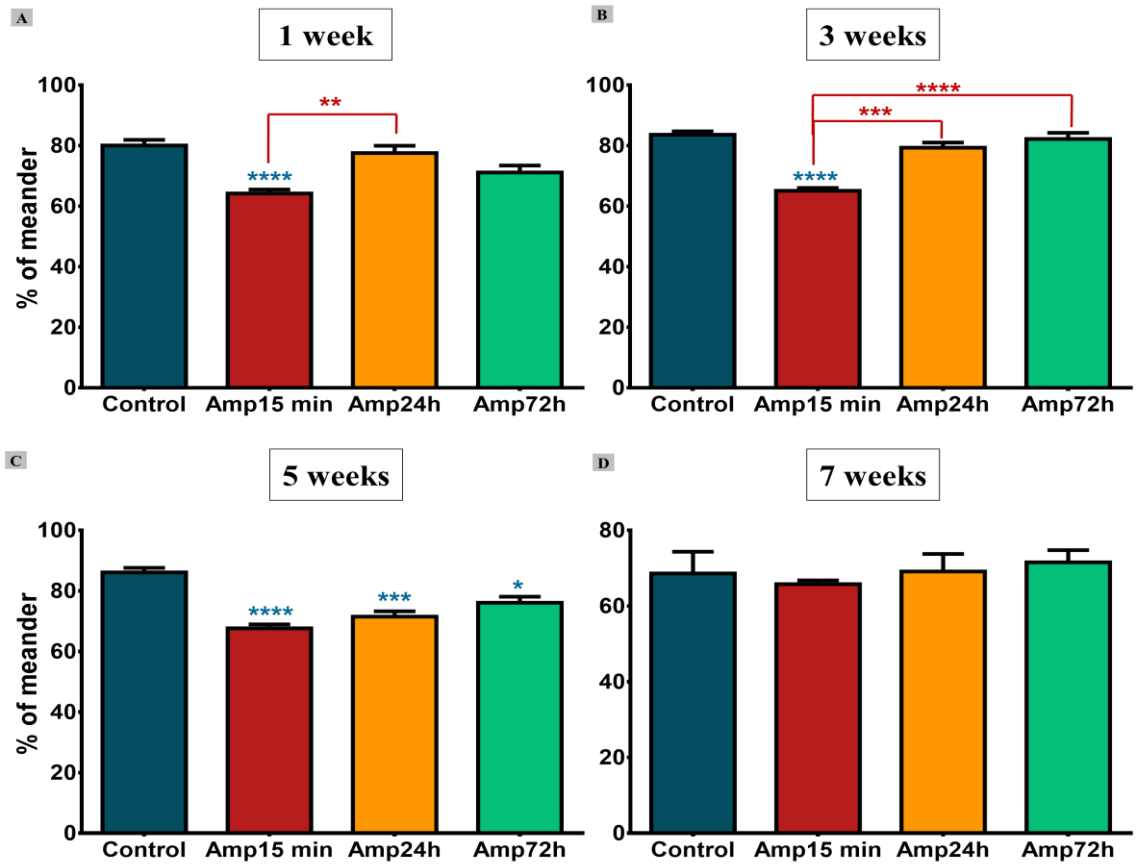

Figure 4.25. Evolution of path straightness (percentage of meander) for each age. The values shown are the mean ± SEM. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, * p-value <0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001. The significant differences in blue are relative to the control group of unamputated flies.

Regarding to the comparison between controls, a gradual increase from 1-week to 5-weeks is observed (see **Figure 4.26**). Although, that increase is not significant. From 5-weeks, the straightness of movement decreased in an abrupt manner. This decrease in straightness can be explained by the normal senescence, which is more evident for healthy flies with 7-weeks.

Relative to the amputation, a same effect was observed for each age. 24h after amputation, the path straightness is similar between 1 and 3-weeks-old flies and between 5 and 7-weeks-old flies. Among 3 and 5-weeks-old, it was verified a slight decrease. In the following time point of recovery (72 hours after amputation), an increase of straightness for 3-weeks-old flies were observed. From 3-weeks, the straightness of path gradually decreases, reaching the control values. According to these results, the motor recovery seems to be more evident 72 hours after amputation (especially for 3-weeks-old flies). Except for 1-week-old flies, the values obtained for the last time point seems to be higher compared to the intermediate time point (24 hours after amputation), although the absence of significant differences.

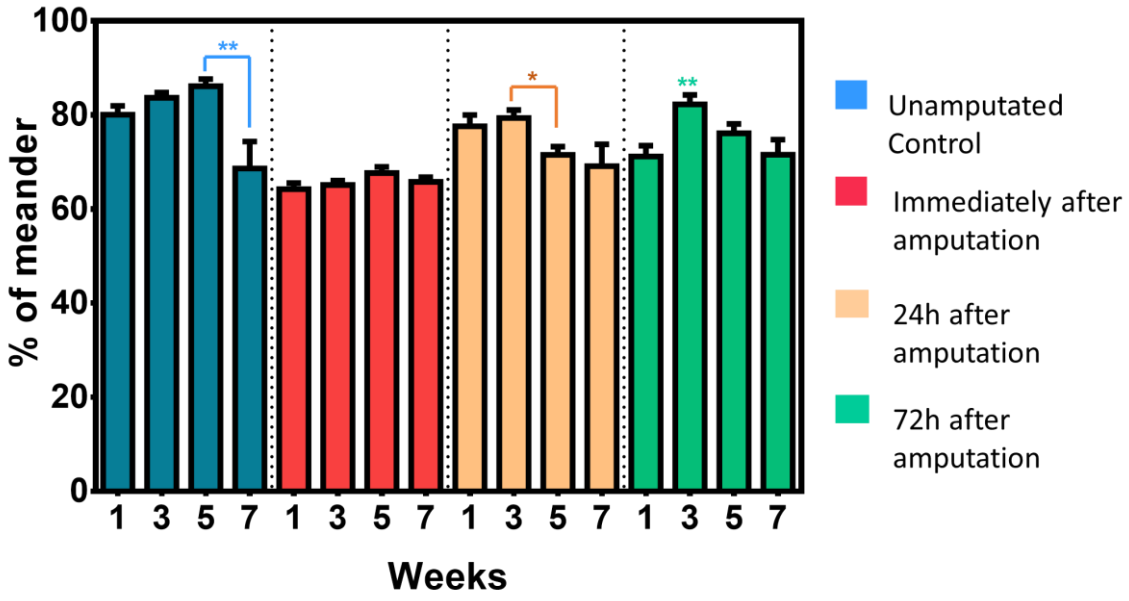

Figure 4.26 General panoramic of evolution of path straightness (percentage of meander) over weeks. The values shown are the mean ± SEM. Statistical analysis with one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis followed by Dunn's post hoc test, * p-value <0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001. The significant differences in blue are relative to the control group of 1-week flies.

In summary, these results confirm the previous quantifications with the *Fly Walker*. Like it was said previously, it was thought that younger flies with 1-week were able to recover from the induced motor injury more quickly and efficiently. However, according to these results, the motor recovery was more evident in flies with 3-weeks. And this efficiently motor recovery can be due to the increase of the total distance traveled and, consequently, the increase of the percentage of locomotor activity. Besides this, it was also verified that these flies can assume higher values for instantaneous speed. In the other age stages, it was verified a gradual decrease in all the activity parameters and in the majority, the decrease is significantly higher to reach the control values (1-week). Once more it was corroborated the hypothesis that the incomplete motor learning presented by 1-week-old flies is similar to the loss of ability to learn new motor skills in 7-weeks-old flies.

CHAPTER 5

Conclusions and Future Work

In this dissertation, the recovery from motor related injuries was studied. For this purpose, it was used *Drosophila melanogaster* as a model. This multi-legged animal was chosen as a model for several reasons. Besides the conserved neural network between them and vertebrates, the fruit fly displays an extremely reliable, stereotyped and stable walking behavior. When flies were exposed to environmental challenges (such as obstacles, load, slope of terrain, among others), they were able to change their kinematic features in order to adapt their locomotion to the new biomechanical challenges. From here, the first question was whether flies can also adapt their locomotion after disease or injury, like the amputation of both middle legs. And test if this reliable walking behavior is dependent on intrinsic factors, like gender and the normal senescence. For this purpose, we used a detailed gait analysis system based on an optical effect, designated by *frustrated Total Internal Reflection* (*fTIR*). This system allowed the quantification of kinematic features with high temporal and spatial resolution. A second question was to test the ability of older animals to recover upon amputation of their middle legs. A final and related question was if there is a relationship between locomotor activity and the motor recovery in younger and older flies.

All the work accomplished to reach the goals previously defined were summarized in **Figure 4.27**. This sequence of procedures was the first step forward towards a better understand of kinematic changes and the cellular mechanisms that mediate motor plasticity and motor recovery upon injury, ultimately setting a blueprint for the cellular pathways used by neuronal circuits to generate new motor pattern, helping to develop or improve new biomedical approaches, like targeted bio-inspired machines to enhance the recovery outcomes.

	Duration (in Months)												
Task	Sep-16	Oct-16	Nov-16	Dec-16	Jan-17	Feb-17	Mar-17	Apr-17	May-17	Jun-17	Jul-17	Aug-17	Sep-17
Learn the techniques and procedures *(e.g.* distinguish males from females, manipulate the FlyWalker and amputation training)													
Training the learned techniques													
Development of a GUI in MATLAB													
Goal 1. Test the gender effect on the normal walking pattern and during the motor recovery													
Video cropping and manual analysis. Data and statistical analysis													
Goal 2. Test the aging effect on normal walking pattern and during the motor recovery													
Preparation and maintenance of aging flies (1, 3, 5 and 7 weeks)													
Video cropping and manual analysis. Data and statistical analysis													
FlyWalker Optimization													
Goal 3. Tracking of motor activity													
Preparation and maintenance of aging flies (1, 3, 5 and 7 weeks)													
Improve FlyMotion													
Data analysis of motor activity													

Figure 4.27 Summary of all work steps developed to achieve the initial goals.

Gender influences the walking pattern and the recovery process after injury

Previous works demonstrated that fruit flies have a reliable and stereotyped walking behavior, that was able to adjust in order to cope new biomechanical challenges, like the amputation of a single front leg (Isakov *et al*., 2016) or single hind leg (Wosnitza *et al.*, 2013). However, the effect of some intrinsic factors, like gender and the aging in normal walking behavior and in motor recovery process remained elusive. For this reason, the kinematic features of locomotion were quantified in male and female healthy flies, but also in injured flies. Firstly, it was verified that in healthy flies, the walking pattern was different for male and female flies.

The first difference encountered was the range of the average speed between the two genders. It was verified that some male flies could achieve higher velocities (above 45 mm/s) and the most used was around 30 - 35 mm/s; whilst in female flies, the most used velocity was around 25 – 30 mm/s (see **Figure 4.4 A**). As a consequence, these differences in the range of average speed caused differences in the percentage of each kind of gait. With the increase of speed, it was corroborated that females used mostly the tripod gait and in a smaller percentage the non-canonical gaits (the tetrapod gait decreases and the wave gait remains mostly unchanged). In contrast, males used less the tripod gait, compensated by the increasing of tetrapod gait in accordance with the increase in the average speed (see **Figure 4.4 B** and **C**).

Despite these results, the higher dependence of most of the walking parameters with speed could mask the differences encountered between males and females. For this reason, the effect of speed was removed through the residual analysis (see Materials and Methods). Considering the residual normalized parameters, we confirmed some significant differences between both genders. Regarding the general walking parameters, it was only identified significant differences in the swing speed and duration. According to these results, males performed the swing phase faster than females and, consequently the swing duration was smaller because they needed to spend less time with the legs in the air. Despite this intuitive relation, these two parameters were not correlated (see **Figure 4.1 A**). These differences could be justified by the anatomical differences, based on smaller size and lower weight of male flies. Taking into account that, they reached higher speeds during locomotion, in addition to perform the swing phase faster than females.

In terms of spatial parameters, there were several significant differences, especially for the AEP footprint clustering, stance straightness and footprint alignment (see **Figure 4.6**). All of these parameters quantify the coordination of movement. The coordination of movement displays different levels of organization: the lowest level is responsible for the continuous interchange of swing and stance phases for each the leg's joints, ensuring the intra-leg coordination. The intermediate level is responsible for the interchanges between all legs, ensuring the inter-leg coordination (Marder and Bucher, 2001). The highest level ensures that both intra- and inter-leg coordination is continuously adapted to the external environment (Berendes *et al*., 2016). Therefore, the AEP footprint clustering quantifies the consistency of footprints of each leg and this consistency can be due to the intra-leg coordination. And according to the results, coordination was higher for males compared with females, which indicates that the footprints of each leg were less clustered. Besides this, the footprint alignment that quantifies the alignment of footprints of each segment was higher for males, which suggests that inter-leg coordination was better in males. Furthermore, stance straightness that measures how wobblier is the movement of legs in contact with the ground relative to the center of the body is lower for males. From these results, it was possible to speculate that females had a better motor control during walking due to the better intra-leg coordination and the less jittery movement. Whilst, in males, the walking movement at higher speeds required a better inter-leg coordination, in order to ensure that there were no errors in choosing the kind

of gait appropriate for the walking velocity. In other words, the increased speed observed for males was done at the expense of a less controlled and wobblier gait.

Additionally, the individual quantification of intra-leg coordination for each leg was made. In this quantification, it was seen that male flies occupied a larger area relative to the body size compared with female flies, indicated by the significant displacement in both directions (x and y directions) (see **Figure 4.7**) In other words, the tarsal contact of male flies tended to be further from the body center. Furthermore, the foreleg contact was shifted anteriorly, whilst the hind leg was shifted posteriorly, in both situations (AEP and PEP). Relative to the middle legs, the AEP position was shifted anteriorly, but the PEP position was shifted posteriorly. These differences may indicate the differential function of each segment of legs and to evidence differences in size and shape of legs between male and female flies (Davis *et al*., 2007).

However, comparing the differences in gait parameters, it was possible to see that there were only differences in the tripod and wave index (see **Figure 4.8 A** and **C**). Like it was verified previously, the tripod gait was less used by the male flies but, in compensation, they used more the wave gait. Interestingly, although males walked with higher speeds, they used preferably the most stable and lowest kind of gait (the wave gait). At the same time, the tripod gait decreased. Additionally, it was verified that presented higher values for duty factor. It suggests that males walked more than females (see **Figure 4.7 E** and **Appendix A.3**).

Besides the kinematic differences founded in the normal walking pattern, it was also founded sexual dimorphisms during the motor recovery process upon removal of the two middle legs. In contrast with the previous studies (Isakov *et al*., 2016; Wosnitza *et al.,* 2013), with the amputation of both middle legs, the postural symmetry was maintained. That ensured that balance of locomotion was not changed and the flies were able learn new kind of gaits, characteristics of quadruped animals. In general, the surgical procedure resulted in an immediate and long-term reorganization of overall posture, single leg kinematics and coordination.

Firstly, it was verified, in most of the variables, that amputation had a similar effect. Only differences in the swing duration (see **Figure 4.12**) and in the step length (see **Figure 4.13 A**) were observed. Regarding the first one, it was observed that swing speed decreased for females and increased for males immediately after amputation, although the absence of significant differences. Relative to the second one, the amputation induced a decrease of step length in females; while, in males, it induced an increase.

Secondly, in the remaining variables, a similar effect was observed for both genders, such as the decrease of movement linearity and of the consistency of footprints (lower intra-leg coordination). In other words, the tarsal contacts became less spatial restricted, causing the spread of footprints (see **Figure 4.9**). Besides this, it was also verified the increase of step period (see **Figure 4.11**) and the swing speed (see **Figure 4.13 B**).

That discrepancy was also seen during the recovery process. Indeed, it was confirmed the gradual motor recovery in most of the walking parameters (*e.g.* AEP footprint clustering and stance straightness) in female flies. While in male flies, the motor recovery suffered oscillations. Another interesting fact, that demonstrated the gradual motor recovery in females compared with males, was the quantification of footfall position of each leg. Once more, in these quantifications, it was possible to see the spread of footprint in relation to the center of body, immediately after the amputation. With the motor recovery, this spread decreased and, consequently the footprints of amputated flies (7 days after the amputation) returned to positions relatively close to the unamputated control. However, this returning was only seen

in the females' case. In males, the spread of footprints is gradually increased. It may indicate a delay in the motor recovery of male flies.

Age affects the walking pattern and the motor recovery process

Like it was said previously, the effect of aging in the normal walking pattern was studied. Besides this, it was studied the effect of amputation in different age stages in order to see if older flies can also recover from motor related injuries. Over the weeks, it was already demonstrated the progressive behavioral decline, especially of motor activity and learning and memory (Grotewiel *et al*., 2005). Besides this, with the present dissertation, was demonstrated the progressive kinematic decline over the weeks, starting from 3-weeks.

With these results was demonstrated the existence of an optimization of motor recovery until 3-weeks. This optimization was verified by the significant existing differences in all the walking parameters comparing with the other age stages, except in the stance straightness. As expected, the linearity of legs' movement relative to the center of the body became lower with advancing age.

Another important confirmation of the kinematic decline with aging was the gradual increase of spread of footprints relative to the center of the body. This increase occurred from 1 to 3-weeks, that rapidly starts to decrease until 7 weeks. This decrease was so abrupt that the footprints obtained for 7-weeks-old flies were positioned close to footprints obtained for the 1-week-old flies (see **Figure 4.16**). So, the normal kinematic adaptation to the normal senescence seems to similar to the initial motor learning in 1-week-old. Indeed, it was demonstrated that for a specific kind of learning (the olfactory memory and learning), the learning became impaired from 10 days (around 1-week) and the memory became impaired from 20 days (around 3-weeks) (Grotewiel *et al*., 2005; Tamura *et al*., 2003). In other words, if we generalize this evidence, flies can learn new motor skills more efficiently until 10-days after, displaying a higher capacity to reorganize the motor maps. However, 20-days after the consolidated memories started to decline. And maybe, this can be the justification of the similarity in the footprints between 1 and 7-weeks-old flies. Beyond this, in other studies was demonstrated that the age-related memory impairments could be observed as defects in intermediate-term memory (ITM) (Tamura et al., 2003) and protein-synthesis-dependent long-term memory (LTM) (Mery, 2007). Specifically, it was verified that ITM became impaired between 20 and 30 days of age and the formation of protein synthesis-dependent LTM increased in early adulthood and subsequently decreasing between 10 and 20 days of age (Tonoki and Davis, 2012). Once more, it was confirmed that motor learning became impaired and, consequently the motor capacity from 3-weeks-old was lost and one possible reason for that is the impairment of ITM. Parallel, from the same age (3-weeks-old) the LTM also became impaired, being its peak in early adulthood (around 1-week). For this reason, some of the motor memories acquired during the first age stage can last until 7-weeks, resulting in similar kinematic properties between 1 and 7-weeks-old flies (see **Figure 4.15 B**, **C** and **E**).

In addition, the worst kinematic performance during aging can be due to the impairments in the muscular system. These impairments include the reducing the length and diameter of nerve branches, the decreasing of synaptic transmission along the giant fiber neuronal circuit and denervation of the neuromuscular junction. All these factors can compromise the normal function of legs during walking (He and Jasper, 2014; Demontis *et al.*, 2013).

Taking into account the previous hypotheses, it can be the justification of the lack of motor recovery after amputation, over the weeks. The absence of motor recovery was most evident in the footprint clustering and in stance straightness (see **Figure 4.17**). In the first one, the amputation had only effects

in 1-week-old flies. Whilst, for the remaining age stages, the footprint clustering did not change with the amputation procedure, remaining mostly unchanged over the recovery period. In other words, the footprints became less clustered with advancing age, ensuring a less intra-leg coordination. In the second one, the amputation caused an abrupt decrease of linearity of movement in all age stages. However, only in 1-weeks-old flies was seen a slight increase of stance straightness during the recovery period. In the remaining age stages, the linearity remained lower and unchanged during the recovery.

The lack of recovery in older flies cannot be explained by lower locomotor activity

One preliminary hypothesis for the lack of motor recovery in older flies was the lower exploratory activity. According to previous studies, the exploratory activity starts to decrease between 14 and 28 days-old (Grotewiel *et al*., 2005). Indeed, in the present thesis, it was demonstrated that the decline of motor activity occurred from 3-weeks-old (around 21 days) in healthy flies (see **Figure 4.19** and **Figure 4.24**). Moreover, we aimed to investigate the lack of motor recovery after amputation. Indeed, by visualizing of path length in an open arena, it was confirmed that amputation induces an immediate decrease of speed (the higher concentration of red circles indicates that the instantaneous speed is lower than 4 mm/s), but also a decrease of motor activity expressed in a smaller distance travelled (see **Figure 4.19**). However, during the recovery period, this effect was dimmed. In other words, the flies could adapt their kinematic features in order to cope the loss of two middle legs and to achieve a more controlled gait. This motor adaptation was observed in 1 and 3-weeks-old flies, but only significant in 3-weeks-old flies. These results are in concordance with the previous conclusions. Additionally, the exploratory activity was statistically similar between 1, 5 and 7-weeks-old flies.

Moreover, these results suggest the existence of an exploratory activity threshold. This minimal activity threshold can be necessary to achieve a concrete motor recovery after injury. For this reason, it is possible to speculate that if flies cannot reach this threshold, the outcomes of motor recovery are not evident (see **Figure 4.24**). Thus, flies with 1, 5 and 7-weeks seems to not reach that threshold to display better outcomes during the recovery process.

Limitations of this work and proposal of possible solutions

The core of this present thesis was to investigate motor recovery after amputation. And according to the results, besides the different effect of amputation in male and female flies, but also in younger and older flies, the motor recovery was previously defined as the returning of each walking parameter to the control values. However, this previous assumption can be considered misleading because the walking pattern during the motor recovery cannot be linearly compared with unamputated flies. One of the reasons for that is the number of legs used during walking and the inter-leg coordination assumed. One possible solution is to define another motor-task that can classify each fly was recovered or not, through a binary variable. After using this binary variable would be possible to construct a logistic regression model to test the significant effect of age and gender in the motor recovery process. Another possibility would be to construct a mathematical model able to modulate theoretically the motor walking pattern of four-legged flies.

Although the behavior and, consequently the kinematic changes are the visible outcomes that underlie the operation of an intricate and complex neural network, the correlation between this and the biological mechanisms was barely considered. For this reason, the further step would be to test some mutant flies, using the several genetic approaches available. One hypothesis would be to test mutants of

learning and memory (such as, rutabaga mutants in which the *rutabaga* gene encodes a calmodulin dependent adenylate cyclase, enzyme that converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) mediating the synaptic plasticity) (McGuire *et al*., 2005) and, then to do the rescue of this gene using the UAS-GAL4 system and in parallel to do the knock-down of this gene in specific brain domains RNA interfere (RNAi) in GAL4 lines and measure the effects on motor recovery upon injury. This approach will facilitate to verify if the motor recovery engages the central brain or the ventral nerve cord.

The force modulation of each leg is another feature that should be considered during the motor recovery in order to understand the differential spreading of footprints during aging (Isakov *et al*., 2016). Besides this and in order to acquire a better understanding of evolution of kinematic adaptation, it would be interesting to analyze the locomotion immediately after eclosion day-by-day until 1-week.

Moreover, we will turn to optogenetics to prevent movement for long periods of time. For this we will inactivate specifically motor neurons in the ventral nerve cord combining a glutamatergic driver (OK371-gal4) with a fragment expressed only in the ventral nerve cord (the homolog of the mammalian spinal cord). By expressing the anion channelrhodopsin GtACR and using this combinatorial system we can prevent moment whenever we expose the flies to green light. We plan to prevent moment for long periods of time after the amputation procedure and test if nevertheless recovery is still achieved. By doing this well will access if a minimal motor activity after amputation will be required for the recovery process.

To conclude, the results presented in this dissertation are relevant towards the better understanding of the effect of gender and aging in motor recovery after injury. Moreover, it reveals the importance of exploratory activity in the motor recovery. Furthermore, the understanding of kinematic features of walking pattern became more complete with the contribution of this present dissertation.

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Appendix A

A.1 Kinematic Parameters: definitions

§[1] indicates definitions described previously by Strauss and Heisenberg 1990 (and references within), §[2] indicates the addition of new definitions previously described by Mendes *et al*., 2013 and Mendes *et al*., 2014

Period§[1]
Time taken to complete one leg cycle consisting of one swing and one stance phase.

Step length§[1]
Distance between two successive footprints of the same leg. No normalization in relation of the direction of propagation.

Stance linearity index§[2]
Average difference between the stance traces generated by each leg during stance phase and a 5-point smoothed line.

Anterior Extreme Position (AEP) (Cruse, 1976)
Position where the leg first contacts the glass after touchdown at the end of swing phase (or protraction).

Posterior Extreme Position (PEP) (Cruse, 1976)
Position at the end of the stance phase, just before the tarsi enter swing phase.

Footprint clustering§[2]
Standard deviation from the average position for all AEPs or PEPs.

Tripod index§[2]
Percentage of frames in a video that display leg combinations defined by the tripod gait.

Tetrapod index§[2]
Percentage of frames in a video that display leg combinations defined by the tetrapod gait.

Appendix A

Gait index§[2]
Average value for a window of frames where tripod configurations = +1 value; tetrapod = −1; and noncanonical = 0.

Footprint alignment§[2]
Standard deviation from the average point of adjacent ipsilateral footprints projected onto the displacement axis. Each set of ipsilateral footprints includes one foreleg, one midleg and one hind leg.

Wave index§[2]
Fraction of frames in a video that display leg combinations defined by the wave (or metachronal) gait (Graham, 1985)

Full stance index§[2]
Fraction of frames in a video in which the body is moving forward while all six legs are in a stance phase.

Tripod duration§[2]
Average duration of a single tripod stance. Only tripod stances that had at least three consecutive frames with the same leg combination were considered.

Inter-tripod time§[2]
Average time to transition from one tripod stance to the next tripod stance. Only tripod stances lasting three or more consecutive frames were considered. In addition, only videos that contained 20% or more of tripod and 40% or less tetrapod configurations were considered.

Duty factor§[2]
Fraction of the step cycle where the leg is in the stance phase (stance duration / period). This parameter allowed to distinguish walks from runs: values ≥0.5 are described as walks, while values below 0.5 are considered runs.

A.2 Principal Component Analysis

The principal component analysis is a multivariate statistical technique that allows to extract the most important information from the initial data. In the present thesis, this analysis was useful to identify a set of relevant variables from the initial data with several parameters and minimize the redundancy. And this new set of variables can be considered relevant to explain the kinematic features of walking pattern. The set of new orthogonal variables is called principal components. Furthermore, this kind of analysis allows to see the pattern of similarity between groups of observations, but also between variables.

A.2.1 Calculation of number of PC considered

Firstly, PCA finds the new variables, the principal components by linear combinations of the original parameters. The first principal component explains the majority of variance of the data and the second component is founded under the constraint of being orthogonal to the first component and to have the largest possible variance. And the remaining components are found in the same way (Jolliffe and Cadima, 2016; Abdi and Williams, 2010; Ringnér, 2008).

The importance of each principal component can be visualized using the *Scree plot* (see **Figure A.2.1**). This plot represents the percentage of variance of data explained by each PC. With this representation was possible to determine the number of components to retain. One way to determine the number of components relevant for the study is to verify the value of each eigenvalue. If the eigenvalue was superior to unit value, the respective principal component is relevant to explain the most of variance than accounted by one of the original variables in standardized data. In this present case, the first five PCs are considered relevant to explain the most variance of the initial dataset (see **Table A.2.1**).

Another way to determine the number of principal components is stipulating previously the satisfactory percentage of variance to explain the total variance. In this case, the first two PCs explain almost 60% of the total variance. For this reason, only the first two PCs were retained to explain the differences between female and male walking kinematics (see **Table A.2.1**).

Another useful representation to see the relationship between all the variables is the *circle of correlations* (Abdi and Williams, 2010) (see **Figure A.2.2**). If the considered variables were positively correlated, they appeared grouped; whilst, if the variables were negatively correlated, their positioning occurs in opposite sides of the circle; in other words, they appeared in opposing quadrants. The distance between the variables and the origin of circle is useful to measure the quality of each variable to interpret the considered components.

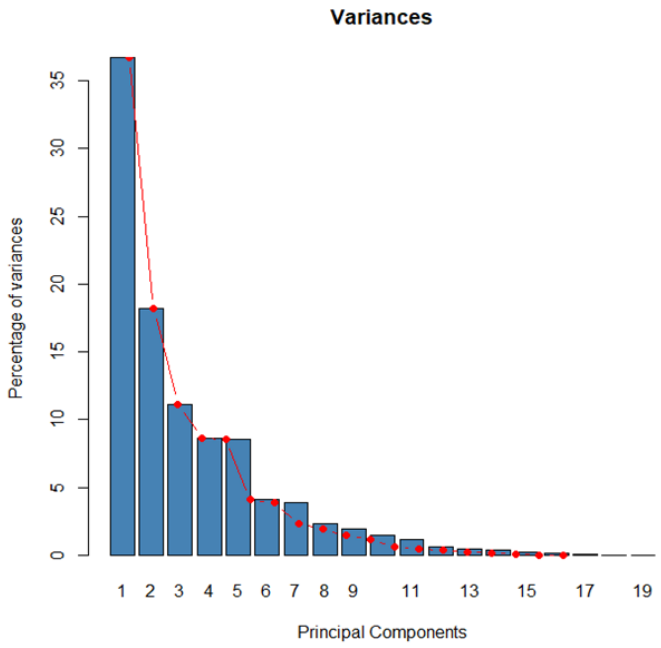

Figure A.2.1. *Scree Plot*, representation of percentage of variance explained by each PC. According to this plot the first five PCs explain almost 80% of total variance. The first two explains almost 60%.

Appendix A

Table A.2.1. Eigenvalues and the variance retained by each principal component.

	Eigenvalue	% Of Variance	Cumulative % Of Variance
PC1	**6.59176**	**34.69349**	**34.69349**
PC2	**4.68778**	**24.67252**	**59.36601**
PC3	**2.39898**	**12.62620**	**71.99221**
PC4	**1.58112**	**8.32167**	**80.31388**
PC5	**1.20933**	**6.36489**	**86.67876**
PC6	0.78511	4.13217	90.81094
PC7	0.52458	2.76093	93.57186
PC8	0.34832	1.83325	95.40512
PC9	0.30580	1.60946	97.01458
PC10	0.25699	1.35258	98.36716
PC11	0.14908	0.78462	99.15178
PC12	0.05762	0.30327	99.45505
PC13	0.05159	0.27153	99.72658
PC14	0.02373	0.12491	99.85149
PC15	0.01370	0.07211	99.92360
PC16	0.01005	0.05289	99.97649
PC17	0.00350	0.01839	99.99488
PC18	0.00097	0.00512	100.00000
PC19	0.00000	0.00000	100.00000

Table A.2.2. Contribution of each variable to the first five components determined by the *squared cosine*.

	PC1	PC2	PC3	PC4	PC5
Speed	0.165258	**0.5761073**	0.076026517	0.004207	0.002284
Step_Frequency	0.066223	**0.8004413**	0.062303527	0.001776	0.003654
Swing_Speed	0.303418	0.2834112	0.1310247	0.001499	0.000686
Step_Length	0.060446	0.01723412	0.068916827	0.454441	0.043599
Step_Period	0.073944	**0.8153481**	0.05177189	0.00216	0.000517
Swing_Duration	0.472147	0.01343653	0.217812891	0.136626	0.022922
Stance_Duration	0.531301	0.03905601	0.171446032	0.038206	0.030632
AEP_FP_Clustering	0.333936	0.1429528	0.032509674	0.125107	0.029717
PEP_FP_Clustering	0.151392	0.01270656	0.062019755	0.362324	0.155478
FP_Alignment	0.475964	0.1079795	0.004437706	0.003388	0.139078
Stance_Straightness	**0.687723**	0.03094582	0.017879818	0.01149	0.075271
Stance_Linearity	**0.609215**	0.06752629	0.010141703	0.019634	0.071949
Tripod_Index	**0.735903**	0.00254537	0.146759065	0.046565	0.035033
Tetrapod_Index	0.18296	0.001169699	0.271989112	0.001477	0.478362
Wave_Index	0.34783	2.14232E-05	0.103673555	0.01948	0.400014
Non_can_Index	0.096763	0.03060231	0.273351507	0.334823	7.3E-05
Duty_Factor	**0.548412**	**0.108222**	0.159765849	0.025754	0.060585
tTripod	**0.365742**	**0.3123459**	0.211956744	0.032009	0.037441
Transition_Time	**0.764896**	0.09209339	0.044062156	0.0233	0.030115

Appendix A

The contribution of each variable to a given principal component can be also quantified. And this contribution is determined by the *squared cosine*. If the *squared cosine* was above 0.5, the respective variable was relevant to explain the specific component. In this case, for example, the tripod index and the transition time are the most relevant variables to explain the PC1; whilst, to explain the PC2 should be considered the step period and the step frequency. Besides this, these two last variables are negatively correlated, that can be observed in the *circle of correlations* by the red color that represents the highest value in the *squared cosine*. All these quantifications and representations were made in R Software.

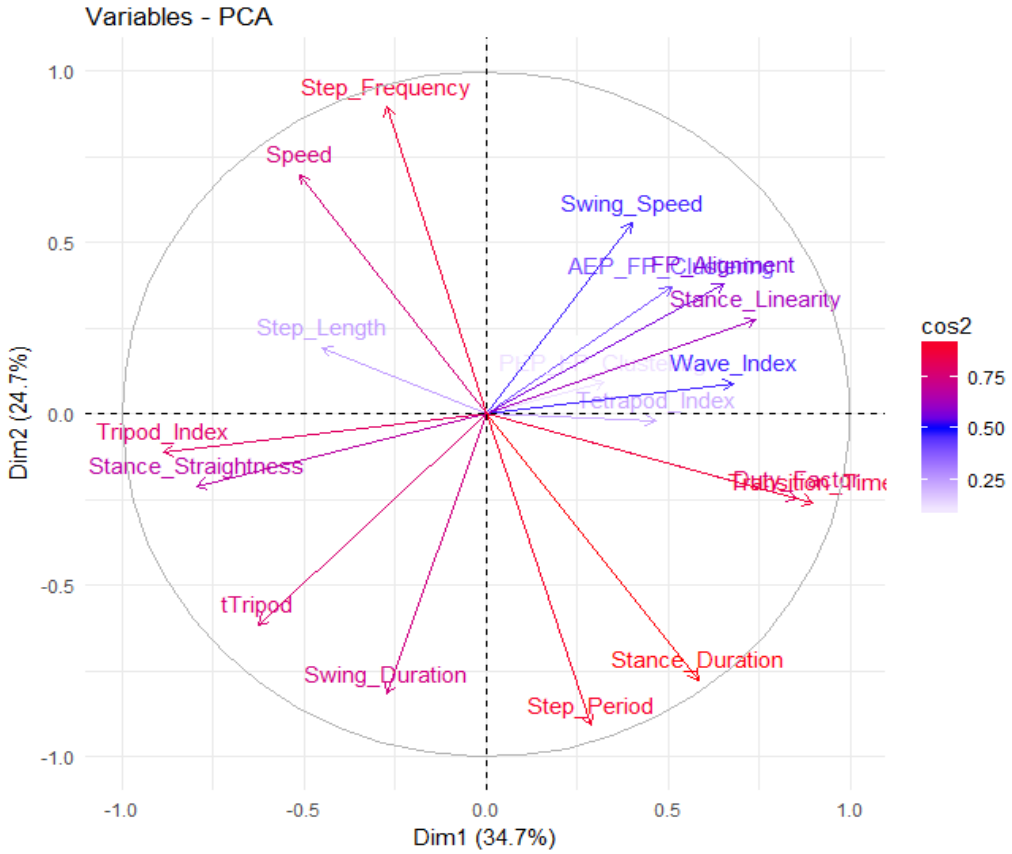

Figure A.2.2. ***Circle of correlations*** **with the specification of the contribution of each variable considered.** According to this plot the red vectors represented variables with a higher contribution to explain the two PCs. Gradually, the darker colors indicate a lower contribution of the respective variable to each PC. Furthermore, the variables that are positively correlated appeared grouped (such as, speed and step frequency); whilst the variables that are negatively correlated appeared on opposite sides of the circle (such as, step frequency and step period). Besides this, the quality of each variable to interpret the two PCs considered can be detected by the distance of vector relative to the origin of the circle.

A.3 Duty Factor: Gender differences

The duty factor is a measure used to distinguish walk from runs, that in previous results demonstrated to be significantly higher for males (see **Figure 4.7 E**). Although the duty factor is highly dependent on velocity, it was verified that regardless of speed, this parameter is a relevant factor in the distinction of walking in females and males. So, in 29% of females analyzed, the duty factor was lower than 0.5, indicating that some flies run instead of walking. So, normally, the preferable kind of locomotion is walking in both genders; however, nevertheless it is mostly used for male flies (see **Figure A.3.1**).

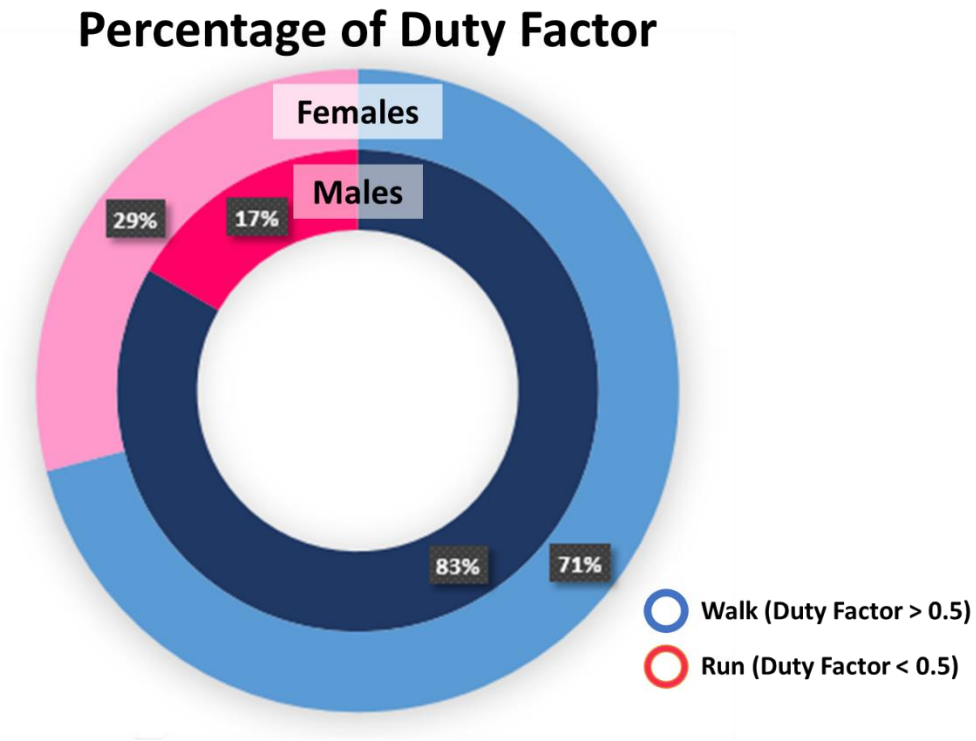

Figure A.3.1. Circular representation of different percentage of duty factor between male and female flies. According to this quantification, normally the female flies have a higher percentage of runs during the tracking normal locomotion. This higher value was quantified by the lower value of duty factor, below the 0.5. However, in both genders the most usual way of locomotion is walking (represented by duty factors above 0.5).

A.4 Lifespan of adult *Drosophila melanogaster*

In order to choose the age stages to study the effect of senescence in the control of walking pattern and during the recover from motor related injury, the curve of lifespan was evaluated. On this curve, it was represented the percentage of survival of adult flies over the weeks and the duration of lifespan, which is around 60 days (around 8 weeks). Clearly, over the weeks the percentage of survival gradually decreases and when it reaches to the 7weeks, the reduction is abrupt. So, the percentage of survival for flies with 7-weeks-old is only 20% (see **Figure A.4.1**).

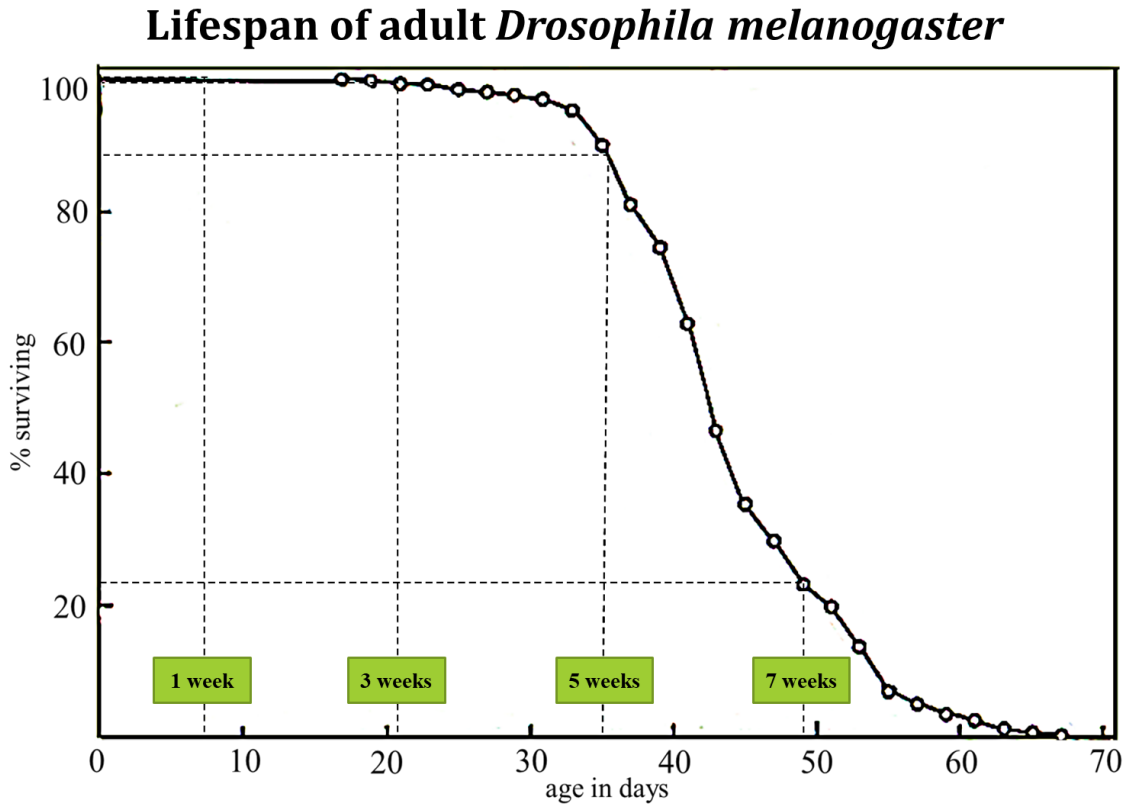

Figure A.4.1. Representation of percentage of survival over the lifespan of adult *Drosophila melanogaster*. Indication of percentage of survival in each age stage considered to evaluate the effect of aging in walking pattern and in the motor recovery after injury. Adapted from Ashburner *et al*., 1979.

A.5 Statistical description of activity parameters

In order to understand the relationship between each activity parameter was created a correlation matrix. According to with this representation, it was observed a strong linear relation between the total distance travelled and the percentage of motor activity Additionally, it was observed a strong linear relation between the two previously mentioned variables and the percentage of meander, which measure the linearity of path (see **Figure A.5.1**).

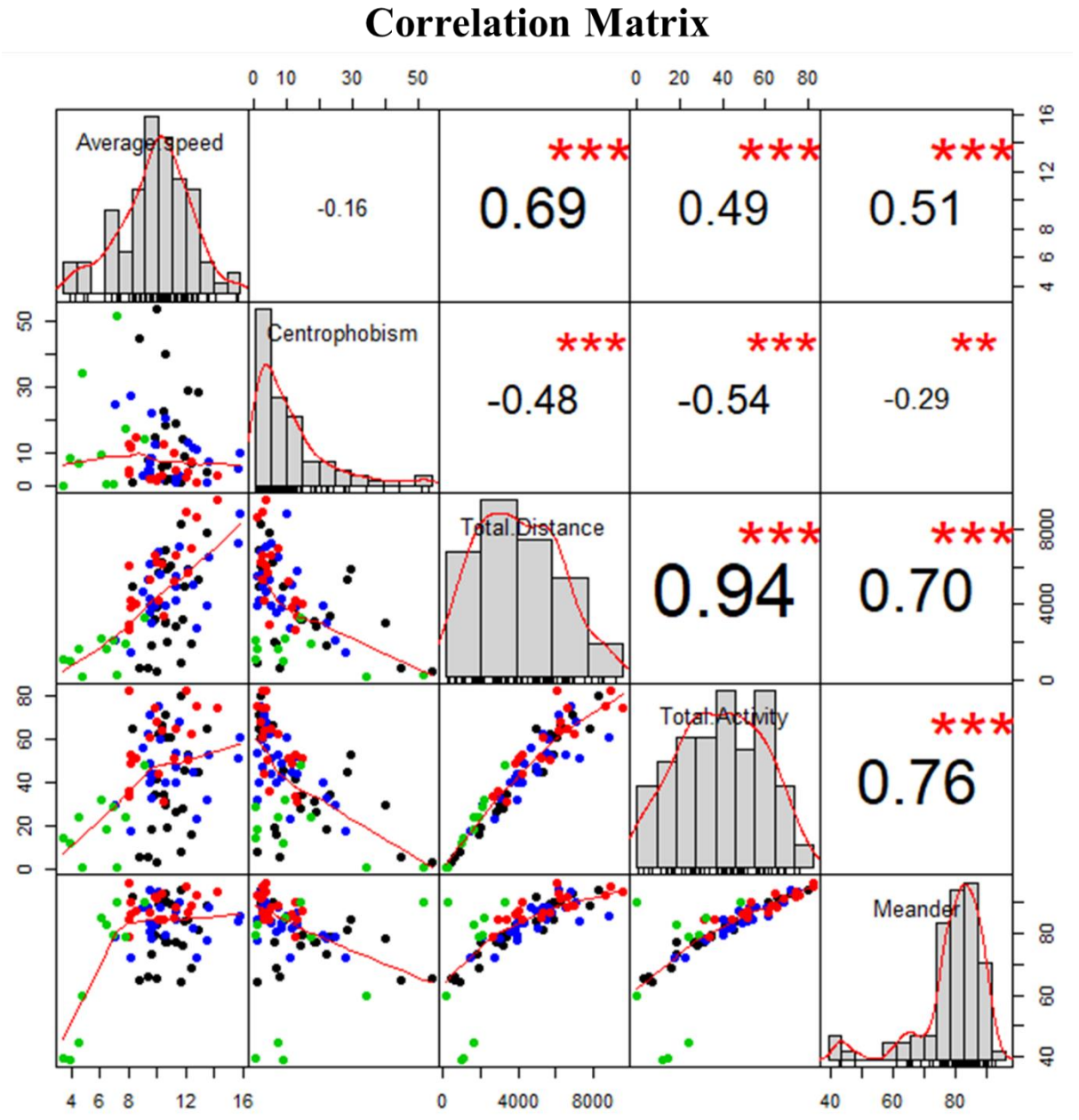

Figure A.5.1. Correlation matrix for activity parameters. In the diagonal of matrix is represented the distribution of each parameter. In the lower panel, the scatter plots for each parameter are represented. Each color represents a specific age (black – 1 week, red – 3 weeks, blue – 5 weeks, green – 7 weeks). In the upper panel are indicated the correlation coefficients between each variable. Each value results the intersection of two variables. The size of the numbers represents the stronger correlation between the specific variables. Statistical analysis preformed in R: * p-value <0.05; ** p-value <0.01; *** p-value <0.001; **** p-value <0.0001